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EXHIBIT "H"

1

Functional Targets

Modification and conjugation techniques are dependent on two interrelated chemical reactions: the reactive functional groups present on the various cross-linking or derivatizing reagents and the functional groups present on the target macromolecules to be modified. Without both types of functional groups being available and chemically compatible, the process of derivatization would be impossible. Reactive functional groups on cross-linking reagents, tags, and probes provide the means to label specifically certain target groups on ligands, peptides, proteins, carbohydrates, lipids, synthetic polymers, nucleic acids, and oligonucleotides. Knowledge of the basic mechanisms by which the reactive groups couple to target functional groups provides the means to design intelligently a modification or conjugation strategy. Choosing the correct reagent systems that can react with the chemical groups available on target molecules forms the basis for successful chemical modification.

The process of designing a derivatization scheme that works well in a given application is not as difficult as it may seem at first glance. A basic understanding of about a dozen reactive functional groups that are commonly present on modification and cross-linking reagents combined with knowledge of about half that many functional target groups can provide the minimum skills necessary to plan a successful experiment.

Fortunately, the principal reactive functional groups commonly encountered on bioconjugate reagents are now present on scores of commercially obtainable compounds. The resource that this arsenal of reagents provides can assist in solving almost any conceivable modification or conjugation problem. The following sections describe the predominant targets for these reagent systems. The functional groups discussed are found on virtually every conceivable biological molecule, including amino acids, peptides, proteins, sugars, carbohydrates, polysaccharides, nucleic acids, oligonucleotides, lipids, and complex organic compounds. A careful understanding of target molecule structure and reactivity provides the foundation for the successful use of all of the modification and conjugation techniques discussed in this book.

1. Modification of Amino Acids, Peptides, and Proteins

Protein molecules are perhaps the most common targets for modification or conjugation techniques. As the mediators of specific activities and functions within living

organisms, proteins can be used *in vitro* and *in vivo* to effect certain tasks. Having enough of a protein that can bind a particular target molecule can result in a way to detect or assay the target, providing the protein can be followed or measured. If such a protein does not possess an easily detectable component, it often can be modified to contain a chemical or biological tracer to allow detectability. This type of protein complex can be designed to retain its ability to bind its natural target, while the tracer portion can provide the means to find and measure the location and amount of target molecules.

Detection, assay, tracking, or targeting of biological molecules by using the appropriately modified proteins are the main areas of application for modification and conjugation systems. The ability to produce a labeled protein having specificity for another molecule provides the key component for much of biological research, clinical diagnostics, and human therapeutics.

In this section, the structure, function, and reactivity of amino acids, peptides, and proteins will be discussed with the goal of providing a foundation of successful derivatization. The interplay of amino acid functional groups and the three-dimensional folding of polypeptide chains will be seen as forming the basis for protein activity. Understanding how the attachment of foreign molecules can affect this tenuous relationship, and thus alter protein function, ultimately will create a rational approach to protein chemistry and modification.

1.1. Protein Structure Reactivity

Amino Acids

Peptides and proteins are composed of amino acids polymerized together through the formation of peptide (amide) bonds. The peptide bonded polymer that forms the backbone of polypeptide structure is called the α-chain. The peptide bonds of the α-chain are rigid planar units formed by the reaction of the α-amino group of one amino acid with the α-carboxyl group of another (Fig. 1). The peptide bond possesses no rotational freedom due to the partial double bond character of the carbonyl-amino amide bond. The bonds around the α-carbon atom, however, are true single bonds with considerable freedom of movement.

The sequence and properties of the amino acid constituents determine protein structure, reactivity, and function. Each amino acid is composed of an amino group

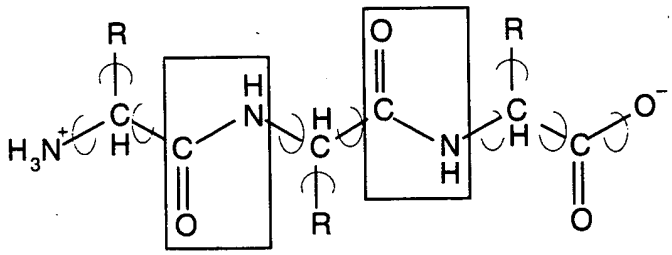


Figure 1 Rigid peptide bonds link amino acid residues together to form proteins. Other bonds within the polypeptide structure may exhibit considerable freedom of rotation.

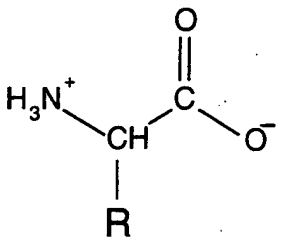


Figure 2 Individual amino acids consist of a primary (α) amine, a carboxylic acid group, and a unique side chain structure (R). At physiological pH the amine is protonated and bears a positive charge, while the carboxylate is ionized and possesses a negative charge.

and a carboxyl group bound to a central carbon, termed the α-carbon. Also bound to the α-carbon is a hydrogen atom and a side chain unique to each amino acid (Fig. 2). There are 20 common amino acids found throughout nature, each containing an identifying side chain of particular chemical structure, charge, hydrogen bonding capability, hydrophilicity (or hydrophobicity), and reactivity. The side chains do not participate in polypeptide formation and are thus free to interact and react with their environment.

Amino acids may be grouped by type depending on the characteristics of their side chains. There are seven amino acids that contain aliphatic side chains that are relatively nonpolar and hydrophobic: glycine, alanine, valine, leucine, isoleucine, methionine, and proline (Fig. 3). Glycine is the simplest amino acid—its side chain consisting of only a hydrogen atom. Alanine is next in line, possessing just a single methyl group for its side chain. Valine, leucine, and isoleucine are slightly more complex with three or four carbon branched-chain constituents. Methionine is unique in that it is the only reactive aliphatic amino acid, containing a thioether group at the terminus of its

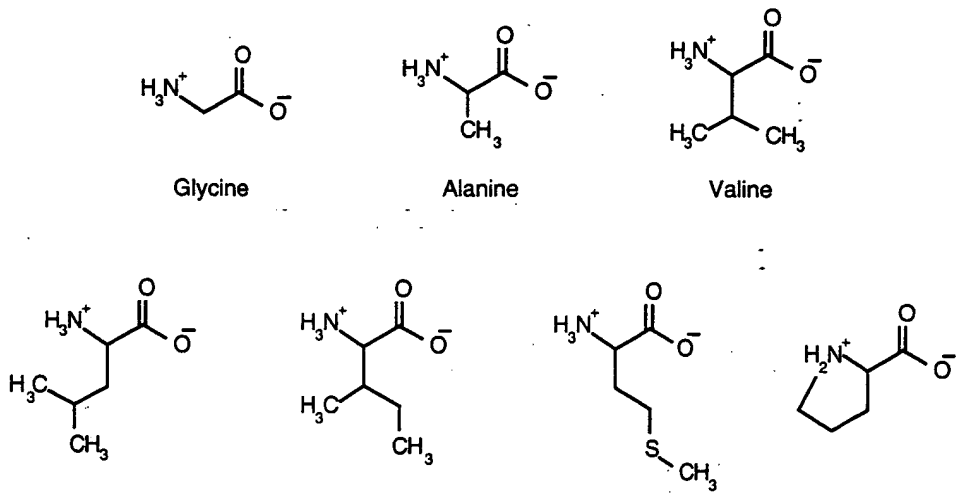


Figure 3 Common aliphatic amino acids.

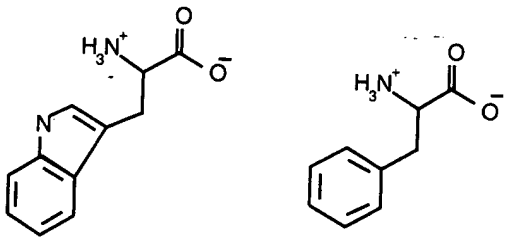
hydrocarbon chain. Proline is actually the only *imino* acid. Its side chain forms a pyrrolidine ring structure with its α-amino group. Thus, it is the only amino acid containing a secondary α-amine. Due to its unique structure, proline often causes severe turns in a polypeptide chain. Proteins rich in proline, such as collagen, have tightly formed structures of high density. Collagen also contains a rare derivative of proline, 4-hydroxyproline, found in only a few other proteins. Proline, however, cannot be accommodated in normal α-helical structures, except at the ends where it may create the turning point for the chain. Poly-proline α-helical structures have been formed, but the structural characteristics of these artificial polypeptides are quite different from native protein helices.

Phenylalanine and tryptophan contain aromatic side chains that, like the aliphatic amino acids, are also relatively nonpolar and hydrophobic (Fig. 4). Phenylalanine is unreactive toward common derivatizing reagents, whereas the indolyl ring of tryptophan is quite reactive, if accessible. The presence of tryptophan in a protein contributes more to its total absorption at 275–280 nm on a mole-per-mole basis than any other amino acid. The phenylalanine content, however, adds very little to the overall absorbance in this range.

All of the aliphatic and aromatic hydrophobic residues often are located at the interior of protein molecules or in areas that interact with other nonpolar structures such as lipids. They usually form the hydrophobic core of proteins and are not readily accessible to water or other hydrophilic molecules.

There is another group of amino acids that contains relatively polar constituents and is thus hydrophilic in character. Asparagine, glutamine, threonine, and serine (Fig. 5) are usually found in hydrophilic regions of a protein molecule, especially at or near the surface where they can be hydrated with the surrounding aqueous environment. Asparagine, threonine, and serine often are found post-translationally modified with carbohydrate in *N*-glycosidic (Asp) and *O*-glycosidic linkages (Thr and Ser). Although these side chains are enzymatically derivatized in nature, the hydroxyl and amide portions have relatively the same nucleophilicity as that of water and are therefore difficult to modify with common reagent systems under aqueous conditions.

The most significant amino acids for modification and conjugation purposes are the ones containing ionizable side chains: aspartic acid, glutamic acid, lysine, arginine, cysteine, histidine, and tyrosine (Fig. 6). In their unprotonated state, each of these side chains can be a potent nucleophile to engage in addition reactions (see the following discussion on nucleophilicity).



Tryptophan Phenylalanine

Figure 4 The two nonpolar aromatic amino acids.

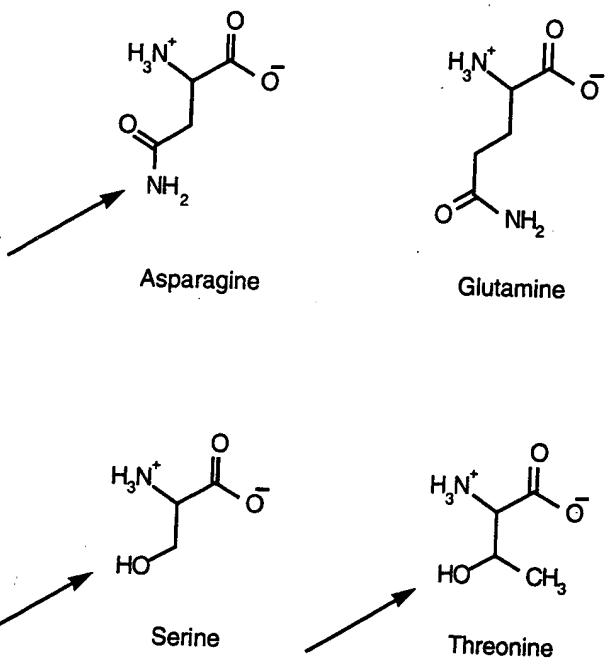


Figure 5 The four polar amino acids. The arrows show the attachment points for carbohydrate residues on glycoproteins.

Both aspartic and glutamic acids contain carboxylate groups that have ionization properties similar to those of the C-terminal α-carboxylate. The theoretical pK_a of the β-carboxyl of aspartic acid (3.7–4.0) and the γ-carboxyl of glutamic acid (4.2–4.5) are somewhat higher than the α-carboxyl groups at the C-terminal of a polypeptide chain (2.1–2.4). At pH values above their pK_a, these groups are generally ionized to negatively charged carboxylates. Thus at physiological pH, they contribute to the overall negative charge contribution of an intact protein (see following section).

Carboxylate groups in proteins may be derivatized through the use of amide bond forming agents or through active ester or reactive carbonyl intermediates (Fig. 7). The carboxylate actually becomes the acylating agent to the modifying group. Amine-containing nucleophiles can couple to an activated carboxylate to give amide derivatives. Hydrazide compounds react in a manner similar to that of amines. Sulfhydryls, while reactive and resulting in a thioester linkage, form unstable derivatives that hydrolyze in aqueous solutions.

Lysine, arginine, and histidine have ionizable amine containing side chains that, along with the N-terminal α-amine, contribute to a protein's overall net positive charge. Lysine contains a straight four-carbon chain terminating in a primary amine group. The ϵ-amine of lysine differs in pK_a from the primary α-amines by having a slightly higher ionization point (pK_a of 9.3–9.5 for lysine versus pK_a of 7.6–8.0 for α-amines). At pH values lower than the pK_a of these groups, the amines are generally protonated and possess a positive charge. At pH values greater than the pK_a, the amines are unprotonated and contribute no net charge. Arginine contains a strongly basic chemical constituent on its side chain called a guanidino group. The ionization point of this residue is so high (pK_a >12.0) that it is virtually always protonated and

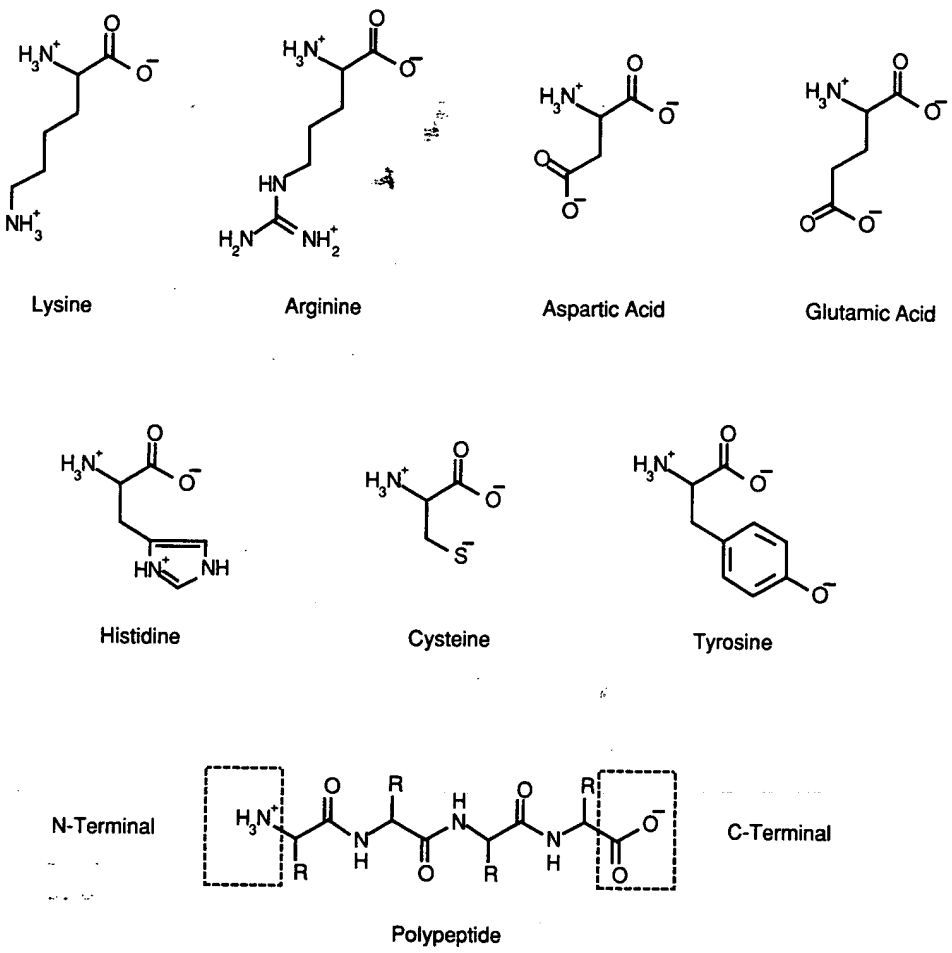


Figure 6 The ionizable amino acids possess some of the most important side-chain functional groups for bioconjugate applications. The C- and N-terminal of each polypeptide chain also is included in this group.

carries a positive charge. Histidine's side chain is an imidazole ring that is potentially protonated at slightly acidic pH values (pK_a 6.7–7.1). Thus, at physiological pH, these residues contribute to the overall net positive charge of an intact protein molecule.

The amine-containing side chains in lysine, arginine, and histidine typically are exposed on the surface of proteins and can be derivatized with ease. The most important reactions that can occur with these residues are alkylation and acylation (Fig. 8). In alkylation, an active alkyl group is transferred to the amine nucelophile with loss of one hydrogen. In acylation, an active carbonyl group undergoes addition to the amine. Alkylating reagents are highly varied and the reaction with an amine nucleophile is difficult to generalize. Acylating reagents, however, usually proceed through a carbonyl addition mechanism, as shown in Fig. 9. The imidazole ring of histidine also is an important reactive species in electrophilic reactions, such as in iodination using radioactive ^{125}I or ^{131}I (Chapter 8, Section 4).

Cysteine is the only amino acid containing a sulfhydryl group. At physiological pH,

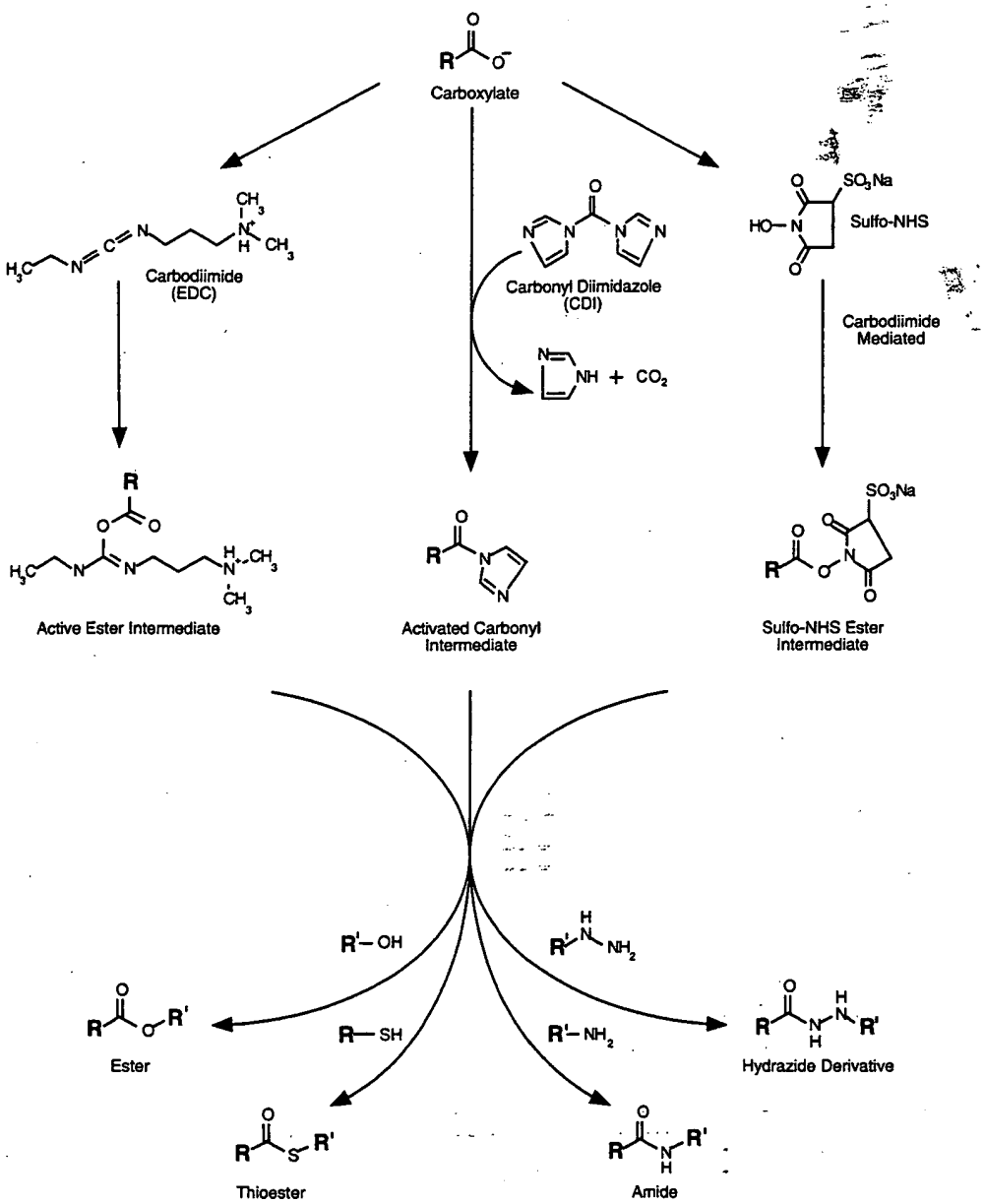


Figure 7 Derivatives of carboxylic acids can be prepared through the use of active intermediates, which react with target functional groups to give acylated products.

this residue is normally protonated and possesses no charge. Ionization only occurs at high pH (pK_a 8.8–9.1) and results in a negatively charged thiolate residue. The most important reaction of cysteine groups in proteins is the formation of disulfide cross-links with another cysteine molecule. Cysteine disulfides (called cystine residues) often are key points in stabilizing protein structure and conformation. They frequently occur between polypeptide subunits, creating a covalent linkage to hold two chains

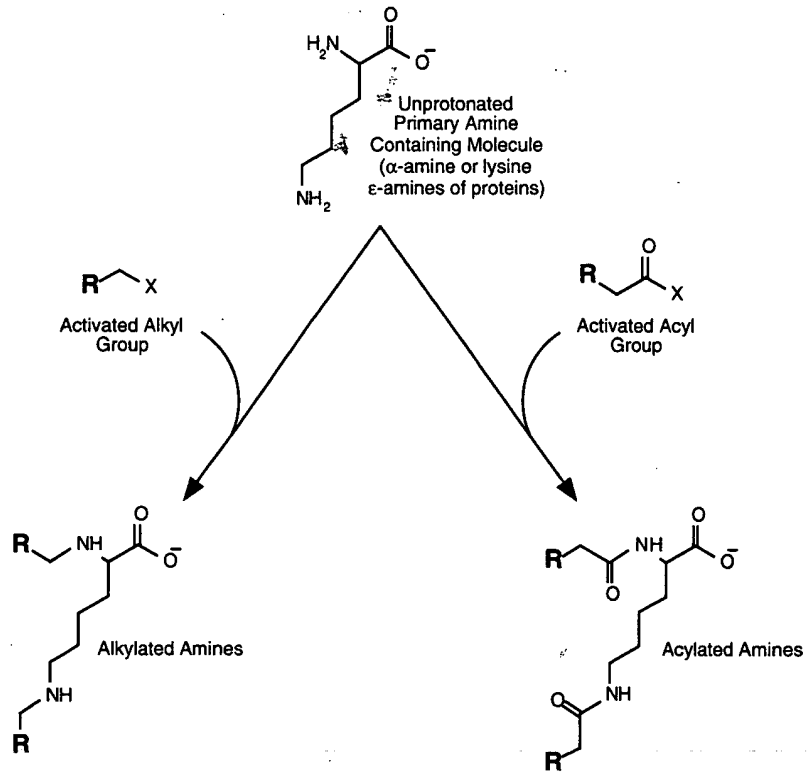


Figure 8 Derivatives of amines can be prepared from acylating or alkylating agents to give amide or secondary amine bonds.

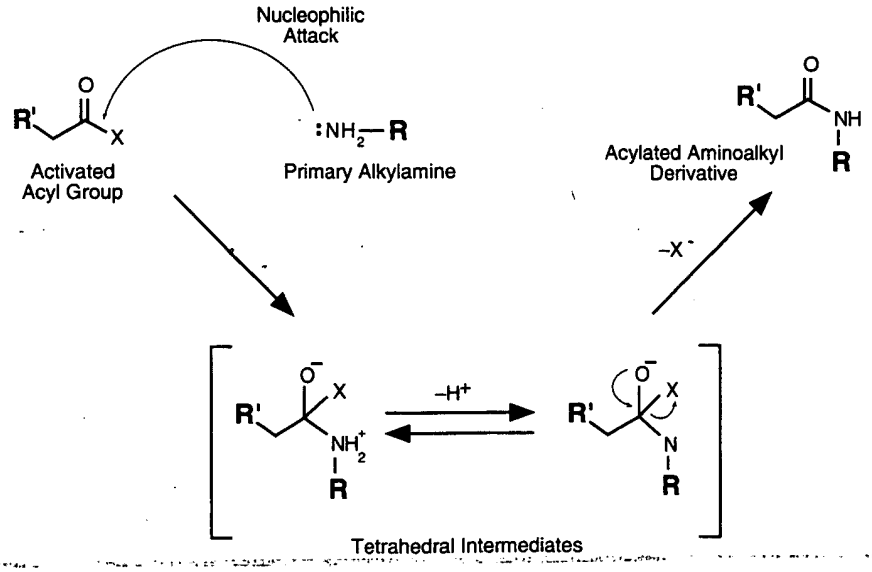


Figure 9 The mechanism of acylation proceeds through the attack of a nucleophile, generating a tetrahedral intermediate, which then goes on to form the product.

together. Cysteine and cystine groups are relatively hydrophobic and usually can be found within the core of a protein. For this reason, it is often difficult to reduce fully the disulfides of large proteins without a deforming agent present to open up the inner structure and make them accessible (see Section 4.1).

Cysteine sulfhydryls and cystine disulfides may undergo a variety of reactions, including alkylation to form stable thioether derivatives, acylation to form relatively unstable thioesters, and a number of oxidation and reduction processes (Fig. 10).

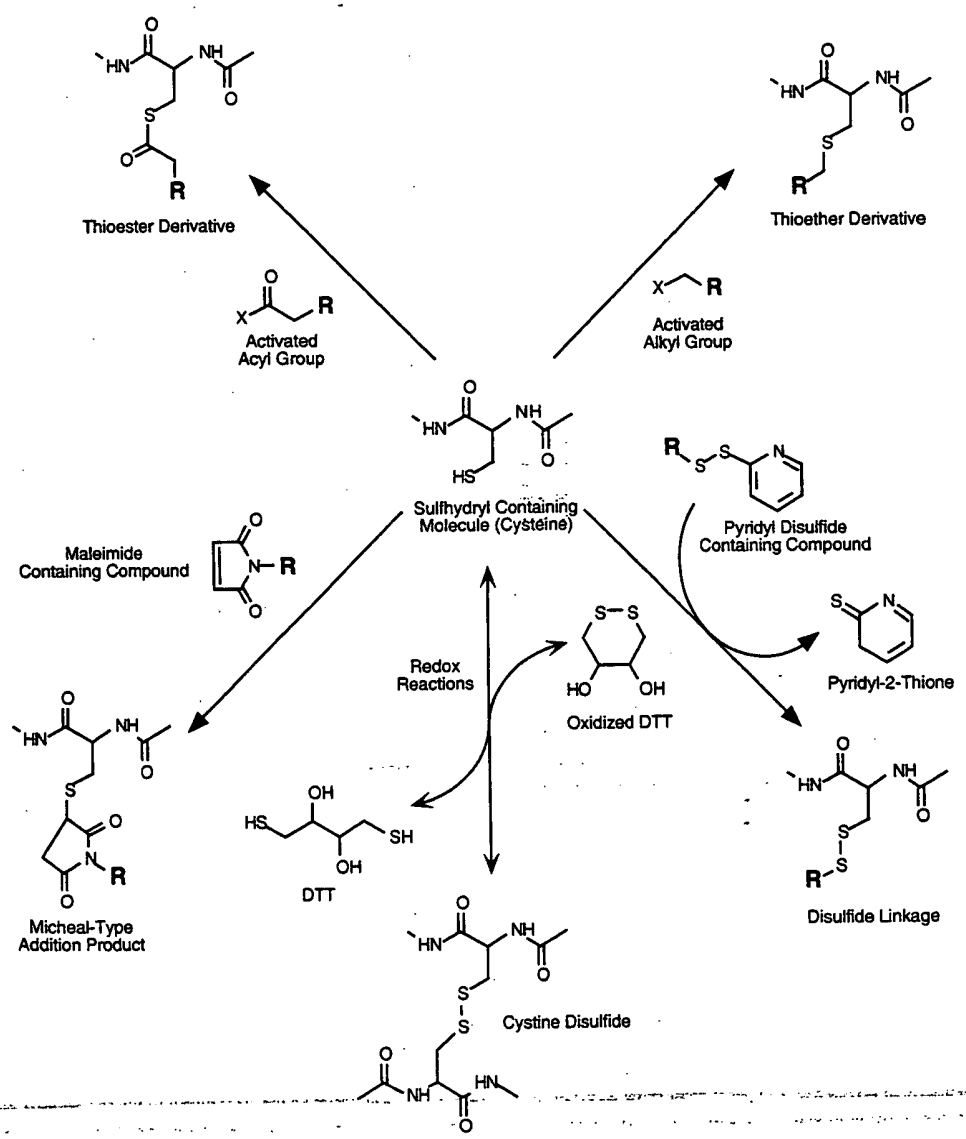


Figure 10 Sulfhydryl groups may undergo a number of additional reactions, including acylation and alkylation. Thiols also may participate in redox reactions, which generate reversible disulfide linkages.

Derivatization of the side chain sulfhydryl of cysteine is one of the most important reactions of modification and conjugation techniques for proteins.

Tyrosine contains a phenolic side chain with a pK_a of about 9.7–10.1. Due to its aromatic character, tyrosine is second only to tryptophan in contributing to a protein's overall absorptivity at 275–280 nm. Although the amino acid is only sparingly soluble in water, the ionizable nature of the phenolic group makes it often appear in hydrophilic regions of a protein—usually at or near the surface. Thus tyrosine derivatization proceeds without much need for deforming agents to further open protein structure.

Tyrosine may be targeted specifically for modification through its phenolate anion by acylation, through electrophilic reactions such as the addition of iodine or diazonium ions, and by Mannich condensation reactions. The electrophilic substitution reactions on tyrosine's ring all occur at the *ortho* position to the —OH group (Fig. 11). Most of these reactions proceed effectively only when tyrosine's ring is ionized to the phenolate anion form.

In summary, protein molecules may contain up to nine amino acids that are readily derivatizable at their side chains: aspartic acid, glutamic acid, lysine, arginine, cysteine, histidine, tyrosine, methionine, and tryptophan. These nine residues contain eight principal functional groups with sufficient reactivity for modification reactions: primary amines, carboxylates, sulfhydryls (or disulfides), thioethers, imidazolyls, guanidinyl groups, and phenolic and indolyl rings. All of these side chain functional groups in addition to the N-terminal α-amino and the C-terminal α-carboxylate form the full complement of polypeptide reactivity within proteins (Fig. 12).

Nucleophilic Reactions and the pI of Amino Acid Side Chains

Ionizable groups within proteins can exist in one of two forms: protonated or unprotonated. Carboxylate groups below their pK_a values exist in the protonated state and are therefore in the conjugate acid form and carry no charge. However, at pH values above the pK_a of the carboxylic group, the acid is ionized and therefore unprotonated to a negative charge. This same relationship is true of the —OH group on the phenol ring of tyrosine. At pH values below its pK_a, tyrosine's side chain is uncharged. Above the pK_a, however, the hydrogen ionizes off, leaving a negatively charged phenolate. Conversely, amine nucleophiles below their pK_a values are in a protonated state and possess a positive charge. At pH values above the pK_a of the amino group, it is then ionized and unprotonated to neutrality.

Each type of ionizable group in proteins will have a unique pK_a based upon the theoretical value for the amino acid and modulated from that value by its own surrounding microenvironment. Minute environmental changes will cause amine containing residues at different structural locations to have different ionization potentials, even if the groups are otherwise chemically identical.

Thus, the actual pK_a of each ionizable group within protein molecules may range considerably lower or higher than the theoretical values as the microenvironment of individual groups changes. Identical side chains in differing parts of a protein molecule may have widely varying pK_a values depending on the immediate chemical milieu. Such factors as the presence of other amino acid side chains in the vicinity, salts, buffers, temperature, ionic strength, and other effects of the solvent medium all play crucial roles in creating microenvironmental changes that affect the ionization potential of these groups (Tanford and Hauenstein, 1956; Schewale and Brew, 1982).

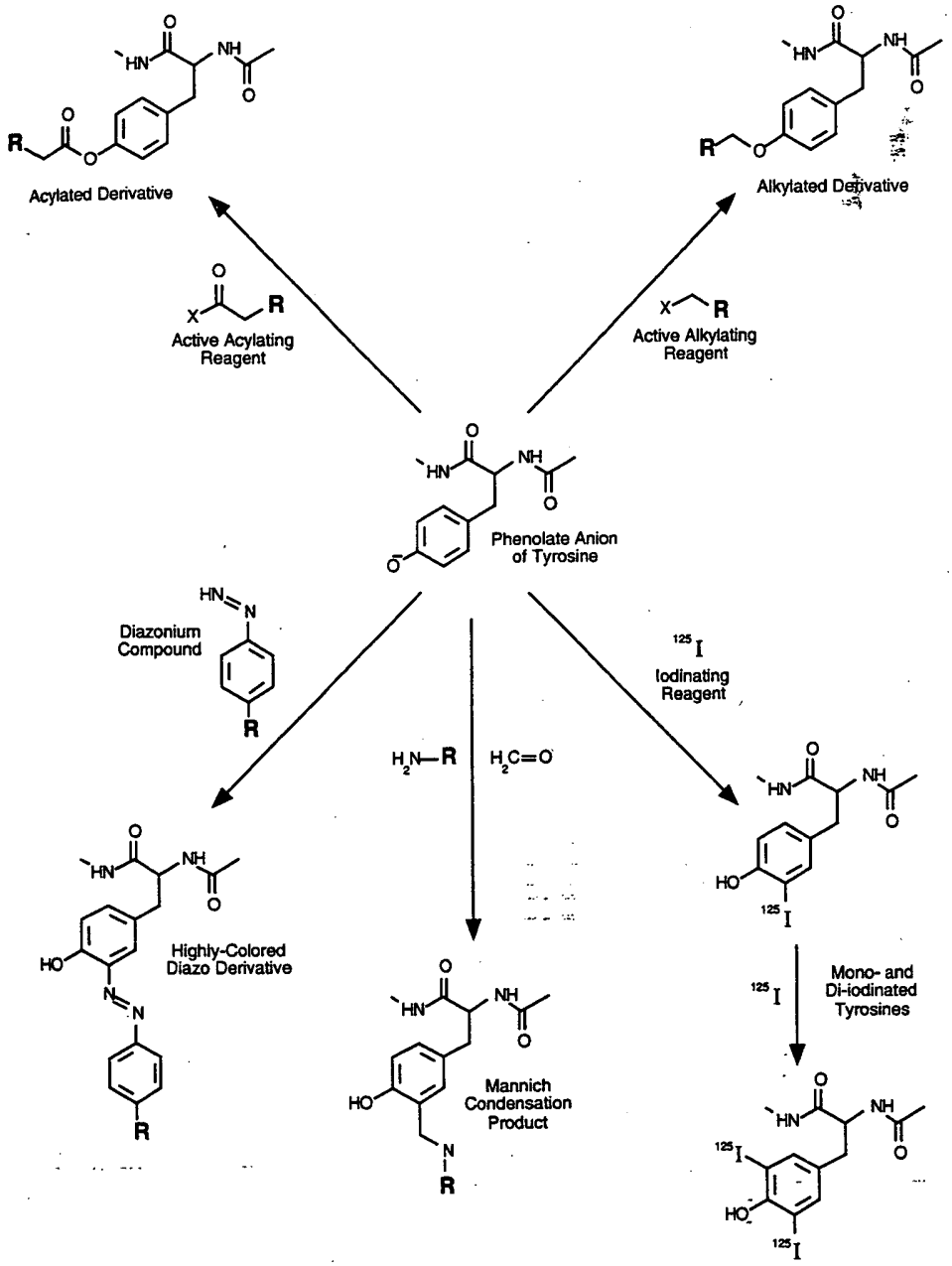


Figure 11 Tyrosine residues are subject to nucleophilic and electrophilic reactions. The phenolate ion may be alkylated or acylated using a variety of bioconjugate reagents. Its aromatic ring also may undergo electrophilic addition using diazonium chemistry or Mannich condensation, or be halogenated with radioactive isotopes such as ^{125}I.

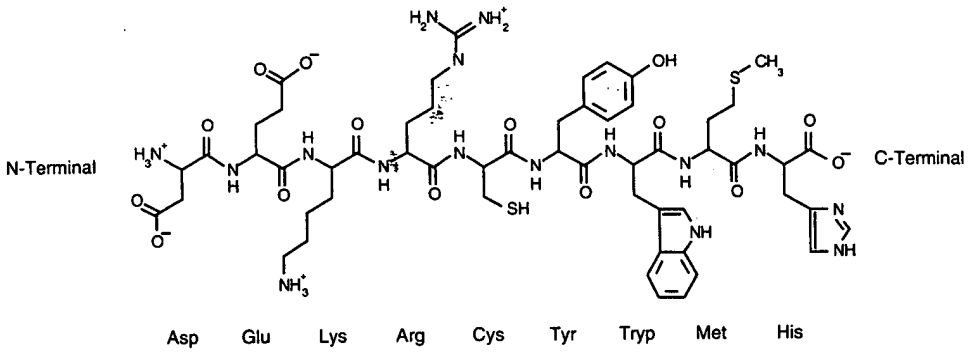


Figure 12 The full complement of polypeptide functional groups is represented by these nine amino acids. Bioconjugate chemistry may occur through the C- and N-terminals of each polypeptide chain, the carboxylate groups of aspartic and glutamic acids, the ε-amine of lysine, the guanidino group of arginine, the sulfhydryl group of cysteine, the phenolate ring of tyrosine, the indol ring of tryptophan, the thioether of methionine, and the imidazole ring of histidine.

The Henderson–Hesselbalch equation (1) explains the relationship of pH and pK_a to the relative ratios of protonated (acid) and unprotonated (base) forms of an ionizable group. Note that the ionized form of such a group does not have to possess a negative charge, as in the case of unprotonated primary amines. Indeed, in that instance it is the protonated amine that bears a charge of positive one. According to the mathematical implications of this equation, an ionizable group at its pK_a value is exactly 50 percent ionized. This means that aspartic acid side chains placed in a medium with a pH equal to its pK_a should have half of its carboxylates ionized to a negative charge and half of them unionized with no charge:

$$pH = pK_a + \log\{[\text{base}]/[\text{acid}]\}. \quad (1)$$

Further implications of this equation are that at one pH unit below or above the pK_a, an ionizable group will be 91% unionized (protonated) or 91% ionized (unprotonated), respectively. Two pH units below or above translate to a 99% unionized or 99% ionized state.

The absolute ratio of protonated-to-unprotonated forms will change from this theoretical approach based upon the microenvironment each group experiences. The reactivity of amino acid side chains is directly related to their being in an unprotonated or ionized state. Many reactions of modification and conjugation occur efficiently only when the nucleophilic species is in an ionized form. As the unprotonated form increases in concentration, the relative nucleophilicity of the ionizable group increases. Many of the reactive groups commonly used for protein modification will couple in greater yield as the pH of the reaction is raised closer to the pK_a of the ionizable target. However, continuing to increase the pH beyond the pK_a may not be necessary for increased yield, and may even be detrimental, because many reactive groups will begin to lose activity through hydrolysis at high pH values.

A nucleophile is any atom containing an unshared pair of electrons or an excess of electrons able to participate in covalent bond formation. Nucleophilic attack at an atomic center of electron deficiency or positive charge is the basis for many of the coupling reactions that occur in chemical modifications. Thus, an uncharged amine

group is a more powerful nucleophile than the protonated form bearing a positive charge. Likewise, a negatively charged carboxylate has greater nucleophilicity than its uncharged, protonated conjugate acid form. In addition, an unprotonated thiolate, bearing a negative charge (RS^-), is a much more powerful nucleophile than its protonated, uncharged sulfhydryl form.

According to the theory of nucleophilicity (Bunnett, 1963; Edwards and Pearson, 1962; Pearson *et al.*, 1968), the relative order of nucelophilicity relative to the major groups in biological molecules can be summarized as follows:

$$R—S^- > R—SH$$

$$R—NH_2 > R—NH_3^+$$

$$R—COO^- > R—COOH$$

$$R—O^- > R—OH$$

$$R—OH = H—OH$$

and finally,

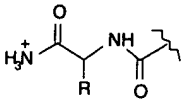
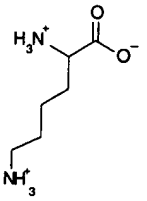
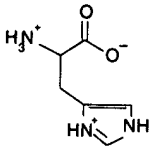
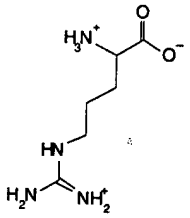
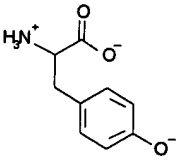
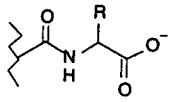
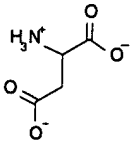
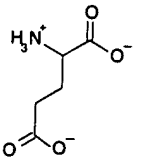
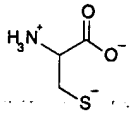
$$R—S^- > R—NH_2 > R—COO^- = R—O^-$$

Using these relationships, it is obvious that the strongest nucleophile in protein molecules is the sulfhydryl group of cysteine, particularly in the ionized, thiolate form. Next in line are the amine groups in their uncharged, unprotonated forms, including the α-amines at the N-terminals, the ϵ-amines of lysine side chains, the secondary amines of histidine imidazolyl groups and tryptophan indole rings, and the guanidino amines of arginine residues. Finally, the least potent nucleophiles are the oxygen-containing ionizable groups including the α-carboxylate at the C-terminal, the β-carboxyl of aspartic acid, the γ-carboxyl of glutamic acid, and the phenolate of tyrosine residues.

According to the theoretical pK_a values for the ionizable side chains of amino acids, nucleophilic substitution reactions involving primary amines or sulfhydryl groups on proteins should not be efficient below a pH of about 8.5 (Table 1). In practice, however, reactions can be done with these groups in high yield at pH values not much higher than neutrality. This discrepancy relates to the changes in pK_a due to microenvironmental effects experienced by the residues within the three-dimensional structure of the protein molecule. In reality, the ϵ-amine groups on lysine side chains with proteins, having theoretical pK_a values over 10, nonetheless exist in sufficient quantity in an unprotonated form even at a pH of 7.2 that modification easily occurs.

One important point should be noted, however. The changes that occur in the pK_a of ionizable groups in protein molecules due to microenvironmental effects make it nearly impossible to select exclusively certain residues for modification simply by careful modulation of reaction conditions. For instance, overlap of the pK_a range for sulfhydryls and amine-containing residues eliminates any chance of directing a reaction solely toward —SH groups by adjusting the pH of the reaction medium. Thus, in practice, for many modification reagents, pH alone cannot be used as an effective modulator of reaction targeting. To site-direct a modification reaction, the proper choice of chemical reactions and reactive groups is far more important than slight changes in pH.

Table 1 pK_a of Ionizable Amino Acids

Group location	Functionality	pK_a range
α-Amine; N-Terminus		7.6–8
Lysine's ϵ-amine		9.3–9.5
Histidine's imidazolyl nitrogen		6.7–7.1
Arginine's guanidinyl group		>12
Tyrosine's phenolic hydroxyl		9.7–10.1
α-Carboxyl; C-terminus		2.1–2.4
Aspartic acid's γ-carboxyl		3.7–4
Gutamic acid's γ-carboxyl		4.2–4.5
Cysteine's sulfhydryl		8.8–9.1

Secondary, Tertiary, and Quaternary Structure

Amino acids are linked through peptide bonds to form long polypeptide chains. The *primary* structure of protein molecules is simply the linear sequence of each residue along the α-chain. Each amino acid in the chain interacts with surrounding groups through various weak, noncovalent interactions and through its unique side chain functional groups. Noncovalent forces such as hydrogen bonding and ionic and hydrophobic interactions combine to create each protein's unique organization.

It is the sequence and types of amino acids and the way that they are folded that provide protein molecules with specific structure, activity, and function. Ionic charge, hydrogen bonding capability, and hydrophobicity are the major determinants for the resultant three-dimensional structure of protein molecules. The α-chain is twisted, folded, and formed into globules, α-helices, and β-sheets based upon the side chain amino acid sequence and weak intramolecular interactions such as hydrogen bonding between different parts of the peptide backbone (Fig. 13). Major secondary structures of proteins such as α-helices and β-sheets are held together solely by massive hydrogen bonding created through the carbonyl oxygens of peptide bonds interacting with the hydrogen atoms of other peptide bonds (Fig. 14).

In addition, negatively charged residues may become bonded to positively charged groups through ionic interactions. Nonpolar side chains may attract other nonpolar residues and form regions of hydrophobicity to the exclusion of water and other ionic groups. Occasionally, disulfide bonds also are found holding different regions of the polypeptide chain together. All of these forces combine to create the *secondary* structure of proteins, which is the way the polypeptide chain folds in local areas to form larger, sometimes periodic, structures.

On a larger scale, the unique folding and structure of one complete polypeptide chain is termed the *tertiary* structure of protein molecules. The difference between

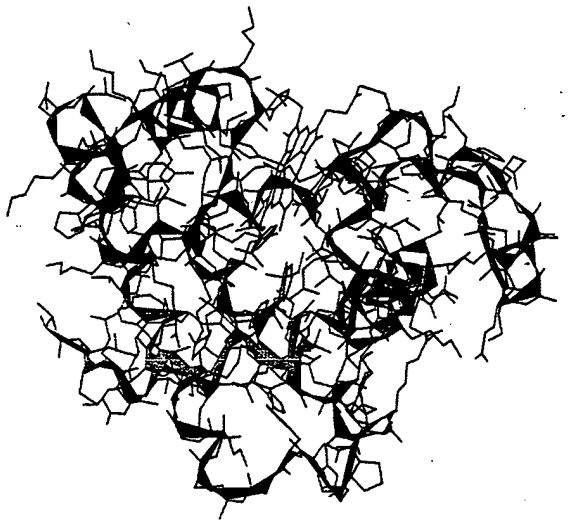


Figure 13 The α-chain structure of myoglobin illustrates the complex nature of polypeptide structure within proteins.

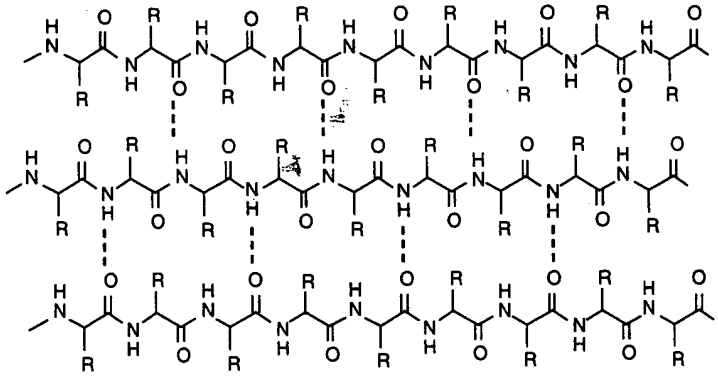


Figure 14 Secondary structure within proteins may be stabilized through hydrogen bonding between adjacent α-chains, forming β-sheet conformations.

local secondary structure and complete polypeptide tertiary structure is arbitrary and sometimes of little practical difference.

Larger proteins often contain more than one polypeptide chain. These multi-subunit proteins have a more complex shape, but are still formed from the same forces that twist and fold the local polypeptide. The unique three-dimensional interaction between different polypeptides in multi-subunit proteins is called the *quaternary* structure. Subunits may be held together by noncovalent contacts, such as hydrophobic or ionic interactions, or by covalent disulfide bonds formed from the cysteine residue of one polypeptide chain being cross-linked to a cysteine sulfhydryl of another chain (Fig. 15).

Thus, aside from the covalently polymerized α-chain itself, the majority of protein structure is determined by weak, noncovalent interactions that potentially can be disturbed by environmental changes. It is for this reason that protein structure can be easily disrupted or denatured by fluctuations in pH or temperature or by substances that can alter the structure of water, such as detergents or chaotropes.

Not surprisingly, chemical modification to the amino acid constituents of a polypeptide chain also may cause significant disruption in the overall three-dimensional structure of a protein. If amino acid residues critical to folding near functionally important regions are modified with chemical groups that change the charge, hydrophilicity, or hydrogen bonding character of the polypeptide chain, protein structure

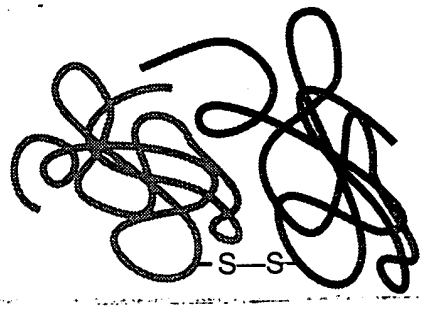


Figure 15 Polypeptide chains may be bound together through disulfide linkages occurring between cysteine residues within each subunit.

may be altered and activity may be compromised. This concept will be discussed further in subsequent sections.

Prosthetic Groups, Cofactors, and Post-translational Modifications

Proteins may contain structures other than polypeptide chains that are important for biological function. Prosthetic groups and cofactors are small organic compounds that are sometimes tightly bound to a protein and aid in forming the active center. A prosthetic group is usually carried within the three-dimensional protein structure in a firm-fitting pocket or even attached through a covalent bond, such as the heme ring associated with cytochrome *c* molecules which is bonded through thioether linkages with adjacent cysteine residues (Fig. 16). Cofactors, by contrast, may be only transiently bound to proteins during periods of activity. Enzymes often require cofactors to act as donors or acceptors of chemical groups that are added to or cleaved from a substrate molecule. Some common cofactors are ATP, ascorbic acid, coenzyme A, NAD, NADP, FAD, FMN, and biotin. Sometimes, the enzyme cofactor also is an energy source for the catalytic reaction, as in the case of ATP-dependent reactions.

Frequently, metal ions are associated with the prosthetic group or cofactor. Heme rings usually contain a chelated iron atom. Occasionally, however, these metals are merely bound within folded polypeptide regions with no additional organic constituents required. Many metal ions are known to participate in enzymatic activity. One or more of the ions of Na, K, Ca, Zn, Cu, Mg, and Mn, as well as Co and Mo are often required by enzymes to maintain activity.

Prosthetic groups and cofactors, whether organic or metallic, may be removed from a protein to create an inactive *apo* protein or enzyme. Loss of these groups may occur through environmental changes, such as removing metal ions from solution or adding denaturants to unfold protein structure. In many cases, full activity can be restored simply by reintroducing the needed group into the surrounding medium.

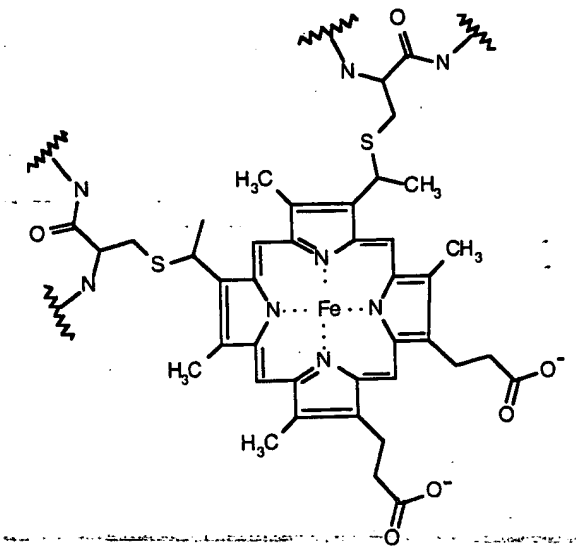


Figure 16 The heme ring of cytochrome *c* is a non-amino acid, prosthetic group bound to the protein through two cysteine residues.

In addition to small organic molecules or metal ions, proteins may have other components tightly associated with them. Nucleoproteins, for instance, contain noncovalently bound DNA or RNA, as in some of the structural proteins of viruses. Lipoproteins contain associated lipids or fatty acids and may also carry cholesterol, as in the high-density and low-density lipoproteins in serum.

During modification or conjugation reactions, prosthetic groups and other associated molecules may be lost or damaged. Metal ions temporarily may be removed by the inclusion of a chelating agent added to maintain sulfhydryl stability during coupling through the —SH groups of a protein. To restore activity after conjugation, it is necessary to remove the chelator and add the required metal salts. Other changes to the prosthetic carriers may not be so easily corrected. For instance, heme-containing molecules are sensitive to the presence of agents that can form a coordination complex with or modify the oxidation state of the chelated metal ion. Some reagent systems may permanently inactivate the heme-containing protein.

Thus, loss of activity can occur not only through changes to the amino acid constituents of a protein, but through prosthetic group or cofactor loss or damage as well. Most of these potential difficulties can be overcome through careful selection of the reaction conditions and through knowledge of the cofactor dependencies that are critical to the activity of the protein being modified.

Post-translational modifications to protein structure are covalent changes that occur as the result of controlled enzymatic reactions or due to chemical reactions not under enzymatic regulation. The most common cellular modification performed on proteins after ribosomal synthesis is glycosylation. Proteins newly synthesized on ribosomes may be transported to the Golgi apparatus where specific glycosyl transferases catalyze the coupling of carbohydrate residues to the polypeptide chains. Glycoproteins and mucoproteins are formed by the coupling of polysaccharides through *O*-glycosidic linkages to serine, threonine, or hydroxylysine and through *N*-glycosidic linkages with the amide side chain group of asparagine.

The structure of most glycoprotein carbohydrate is branched with the sugars mannose, *N*-acetyl glucosamine, sialic acid, glactose, and L-fucose being prevalent. Asparagine-linked polysaccharides are well characterized and are known to be constructed of a core unit consisting of three mannose residues and 2 *N*-acetyl glucosamine (GlcNAc) residues. The GlcNAc residues are bound to the Asp side chain amide nitrogen through a β1 linkage (Kornfield and Kornfield, 1985). The three mannose groups then usually form the first branch point in the oligosaccharide chain (Section 2).

The content by weight of carbohydrate in glycoproteins may vary from only a few percent to over 50% in some proteins in mucous secretions. Although the function of the polysaccharide in most glycoproteins is unknown, in some case it may provide hydrophilicity, recognition, and points of noncovalent interaction with other proteins through lectin-like affinity binding.

The presence of carbohydrate on protein or peptide molecules provides important points of attachment for modification or conjugation reactions. Coupling only through polysaccharide chains often can direct the reaction away from active centers or critical points in the polypeptide chain, thus preserving activity. Polysaccharides can be specifically targeted on glycoproteins through mild sodium periodate oxidation. Periodate cleaves adjacent hydroxyl groups in sugar residues to create highly reactive aldehyde functionalities (Section 4.4). The level of periodate addition can be adjusted to cleave selectively only certain sugars in the polysaccharide chain. For instance, a concentration of 1m*M* sodium periodate specifically oxidizes sialic acid residues to

aldehydes, leaving all other monosaccharides untouched. Increasing the concentration to 10 m*M*, however, will cause oxidation of other sugars in the carbohydrate chain, including galactose and mannose. The generated aldehydes then can be used in coupling reactions with amine- or hydrazide-containing molecules to form covalent linkages. Amines can react with formyl groups under reductive amination conditions using a suitable reducing agent such as sodium cyanoborohydride. The result of this reaction is a stable secondary amine linkage (Chapter 2, Section 5.3). Alternatively, hydrazides spontaneously react with aldehydes to form hydrazone linkages, although the addition of a reducing agent increases the efficiency of the reaction (Chapter 2, Section 5.1).

Another form of post-transitional modification that may add carbohydrate to a polypeptide is nonenzymatic glycation. This reaction occurs between the reducing ends of sugar molecules and the amino groups of proteins and peptides. See Section 2.1 for further details and the reaction sequence behind this modification.

Protecting the Native Conformation and Activity of Proteins

The goal of most protein modification or conjugation procedures is to create a stable product with good retention of the native state and activity. Ideally, any derivatization should result in a protein that performs exactly as it would in its unmodified form, but with the added functionality imparted by whatever is conjugated to it. Thus, an antibody molecule tagged with a fluorophore should retain its ability to bind to antigen and also have the added function of fluorescence.

One of the best ways to ensure retention of activity in protein molecules is to avoid chemical reaction at the active center. The active center is that portion of the protein where ligand, antigen, or substrate binding occurs. In simpler terms, the active center (or active site) is that part that has specific interaction with another substance (Means and Feeney, 1971). For the preparation of enzyme derivatives, it is important to protect the site of catalysis where conversion of substrate to product occurs. When working with antibody molecules, it is crucial to stay away from the two antigen-binding sites.

The best chemical procedures avoid the active site by selecting functional groups away from that area or by protecting the site through the incorporation of additives. In some cases, the inclusion of substrates, cofactors, ligands, inhibitors, or antigens in the modification reaction will protect the active site. Addition of the appropriate substance can bind the active site and mask it from modification by cross-linking agents. In enzyme derivatization procedures, this is often just a matter of adding a reversible inhibitor or substrate analog. For instance, when working with alkaline phosphatase merely doing the reaction in phosphate buffer protects the active center from chemical modification, since phosphate ions bind in the catalytic site. With trypsin, the incorporation of benzamidine similarly masks and protects the active site.

However, protecting the antigen-binding sites on an antibody molecule by using this method is often more difficult. Inclusion of antigen to mask the binding sites is effective in blocking these areas, but it also may cause irreversible cross-linking of the antigen to the antibody. This is especially true when the antigen is a peptide or a protein having the same chemical reactive groups as the antibody. Any modification reactions that are directed at the antibody may modify the antigen as well. Therefore, only use this method if the antigen is lacking in the chemical targets that are going to be used on the antibody. For instance, if the polysaccharide chains on the antibody are to be modified, then using a protein antigen that does not contain carbohydrate to block the antigen binding sites may work well.

An equally effective method of protecting the activity of a protein is by using site-directed reactions that result in modifications away from the active center. In some cases, specific functionalities are known to be present only at restricted sites within the three-dimensional structure of a protein. If these functional groups are not present close to the active site, then using them exclusively for modification reactions should ensure good retention of activity.[3] For instance, sulfhydryl groups or carbohydrate chains are often present in limited quantity and in specific regions on a protein. Selecting reagent systems that target these groups ensures derivatization only at restricted sites within the protein molecule, thus potentially avoiding the active center.

Surprisingly, the goal of some protein modification or conjugation schemes is to somewhat alter the native presentation of the product. This is especially true in hapten–carrier conjugation, as used for immunogen or vaccine production. In this case, the main objective is to modify the environment of the hapten to create an immunological response *in vivo*. A hapten is usually a small molecule that is not able to generate an immune response on its own, but can react with the products of such a response once generated. Most often these products are antibodies having specificity for the hapten.

The complexities involved in achieving a successful conjugation strategy are best illustrated in the problems and concerns dealing with hapten–carrier conjugation. In order to produce the initial immune response to a small molecule, the hapten is typically coupled to a larger protein that can generate a response on its own. In simple terms, the larger carrier protein confers immunogenicity to the smaller hapten. The native presentation of the hapten is altered toward the immune system, thus creating the immune response.

The site of attachment of the hapten to the carrier and the nature of the cross-linker are both important to the specificity of the resultant antibodies generated against it. For proper recognition, the hapten must be coupled to the carrier with the appropriate orientation. For an antibody to recognize subsequently the free hapten without carrier, the hapten–carrier conjugate must present the hapten in an exposed and accessible form. Optimal orientation is often achieved by directing the cross-linking reaction to specific sites on the hapten molecule. With peptide haptens, this is typically done by attaching a terminal cysteine residue during synthesis. This provides a sulfhydryl group on one end of the peptide for conjugation to the carrier. Cross-linking through this group provides hapten attachment only at one end, therefore ensuring consistent orientation.

In hapten–carrier conjugation, the goal is not to maintain the native state or stability of the carrier, but to present the hapten in the best possible way to the immune system. In reaching this goal, the choice of conjugation chemistry may control the resultant titer, affinity, and specificity of the antibodies generated against the hapten. It may be important in some cases to choose a cross-linking agent containing a spacer arm long enough to present the antigen in an unrestricted fashion. It also may be important to control the density of the hapten on the surface of the carrier. Too little hapten substitution may result in little or no response. A hapten density too high actually may cause immunological suppression and decrease the response. In addition, the cross-linker itself may generate an undesired immune response. Fortunately, for the majority of hapten–carrier conjugation problems, a few main cross-linking techniques provide a workable compromise to solving all these concerns and ultimately generating an effective immune response (Chapter 9).

1.2. Protein Cross-linking Methods

The cross-linking of two proteins using a simple homobifunctional reagent (Chapter 4) potentially can result in a broad range of conjugates being produced (Avrameas, 1969). The reagent initially may react with either one of the proteins, forming an active intermediate. This activated protein may then form cross-links with the other protein or with another molecule of the same protein. The activated protein also may react intramolecularly with other functional groups on part of its own polypeptide chain. Other cross-linking molecules may continue to react with these conjugated species to form various mixed products, including severely polymerized proteins that may fall out of solution (Fig. 17).

The problems of indeterminate conjugation products are amplified in single-step reaction procedures using homobifunctional reagents (Chapter 4). Single-step procedures involve the addition of all reagents at the same time to the reaction mixture. This technique provides the least control over the cross-linking process and invariably leads

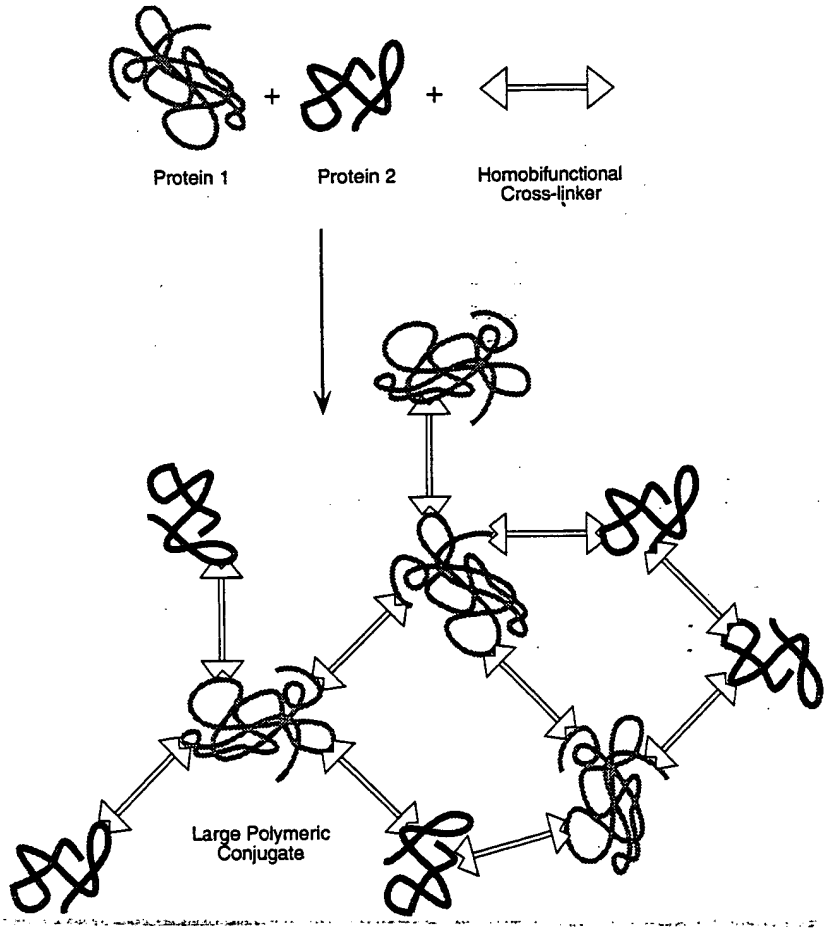


Figure 17 Protein cross-linking reactions done using homobifunctional reagents can result in large polymeric complexes of multiple sizes and indefinite structure.

to a multitude of products, only a small percentage of which represent the desired or optimal conjugate. Excessive conjugation may cause the formation of insoluble complexes that consist of very high molecular weight polymers. For example, one-step glutaraldehyde conjugation of antibodies and enzymes (Chapter 10, Section 1.2) often results in significant oligomers and precipitated conjugates. To overcome this shortcoming, multistep reaction procedures have been developed using both homobifunctional and heterobifunctional reagents (Chapter 5). Controlled, multistep conjugation protocols alleviate the polymerization problem and form relatively low molecular weight, soluble antibody–enzyme complexes (Chapter 10, Section 1.1).

In two-step protocols, one of the proteins to be conjugated is reacted or "activated" with a cross-linking agent and excess reagent and by-products are removed. In the second stage, the activated protein is mixed with the other protein or molecule to be conjugated, and the final conjugation process occurs (Fig. 18).

The use of homobifunctional reagents in two-step protocols still creates many of the problems associated with single-step procedures, because the first protein can cross-link and polymerize with itself long before the second protein is added. Homobifunctional reagents by definition have the same reactive group on either end of the cross-linking molecule. Since the protein to be activated has target functional groups on every molecule that can couple with the reactive groups on the cross-linker, both ends of the reagent potentially can react. This inherent potential to polymerize uncontrollably unfortunately is characteristic of all homobifunctional reagents, even in multistep protocols.

The greatest degree of control in cross-linking procedures is afforded using heterobifunctional reagents (Chapter 5). Since a heterobifunctional cross-linker has different reactive groups on either end of the molecule, each side can be specifically directed toward different functional groups on proteins. Using a multistep conjugation protocol with a heterobifunctional reagent can allow one macromolecule to be activated, excess cross-linker removed, and then a second macromolecule added to induce the final linkage. Directed conjugation will occur as long as the first protein activated does not have groups able to couple with the second end of the cross-linker, whereas the second molecule does possess the correct functionalities.

Occasionally, the second protein does not naturally have the target groups necessary to couple with the second end of the cross-linker. In such cases, a specific functional group usually can be created to make the conjugation successful (Section 4). In such three-step systems, the first protein is activated with the heterobifunctional reagent and purified away from excess cross-linker. The second protein is then modified to contain the specific target groups required for the second stage of the conjugation. Finally in step three, the two modified proteins are mixed to cause the coupling reaction to happen (Fig. 19).

Two- and three-step protocols using heterobifunctional cross-linkers often are designed around amine-reactive and sulfhydryl-reactive chemical reactions. Many of these reagents utilize NHS esters on one end for coupling to amine groups on the first protein and maleimide groups on the other end that can react with sulfhydryls on the second protein. The NHS ester end is reacted with the first protein to be conjugated, forming an activated intermediate containing reactive maleimide groups. Fortunately, the maleimide end of such cross-linkers is relatively stable to degradation; thus the activated protein can be isolated without loss of sulfhydryl coupling ability. Additionally, if the second protein does not contain indigenous sulfhydryls, these can be created by an abundance of methods (Section 4.1). After mixing the maleimide-

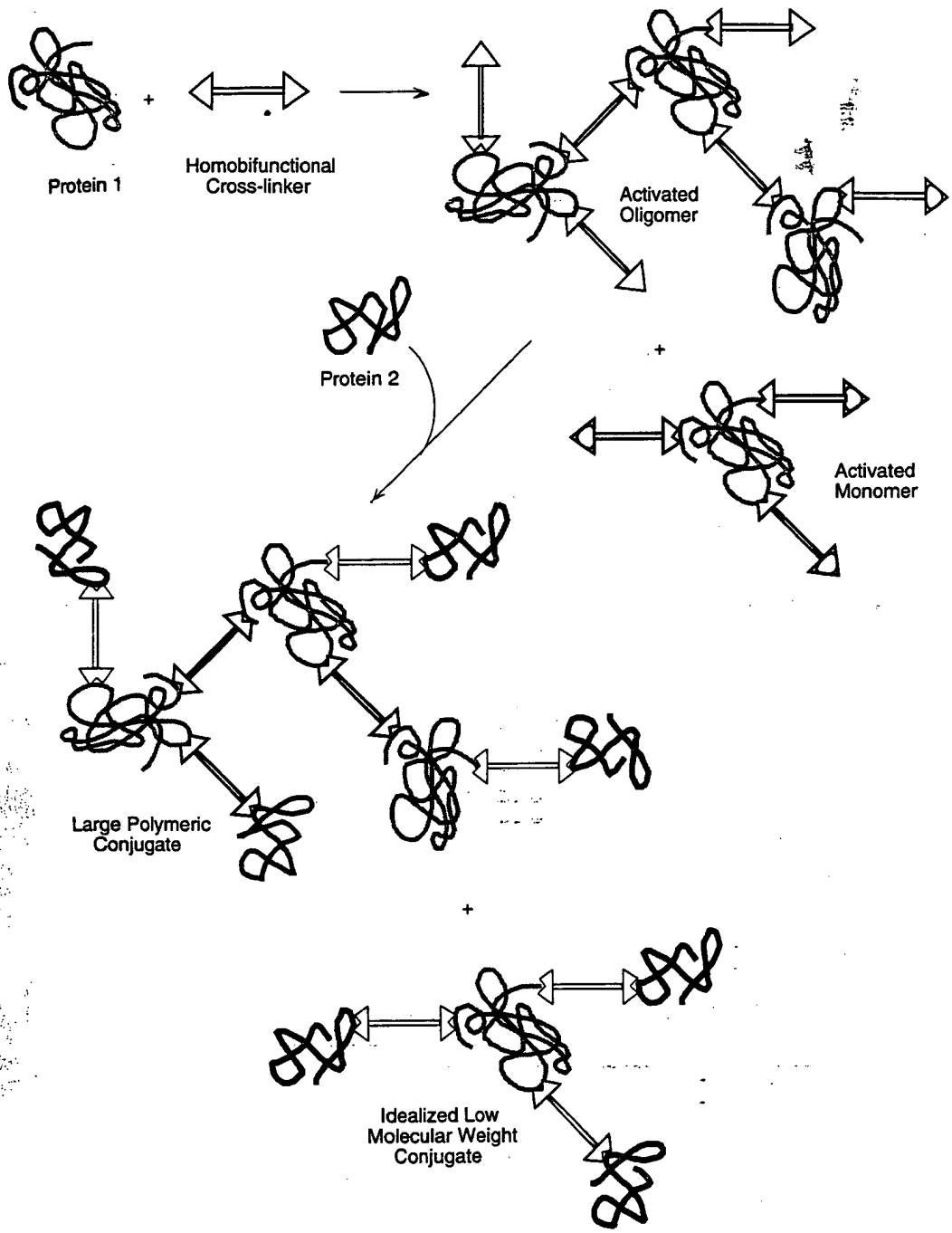


Figure 18 A two-step protocol using a homobifunctional cross-linking agent offers more control than single step methods, but still may result in oligomer formation.

activated protein with the sulfhydryl-containing protein, conjugation can occur only in one direction.

Control of the products of conjugation increases as the protocols progress from

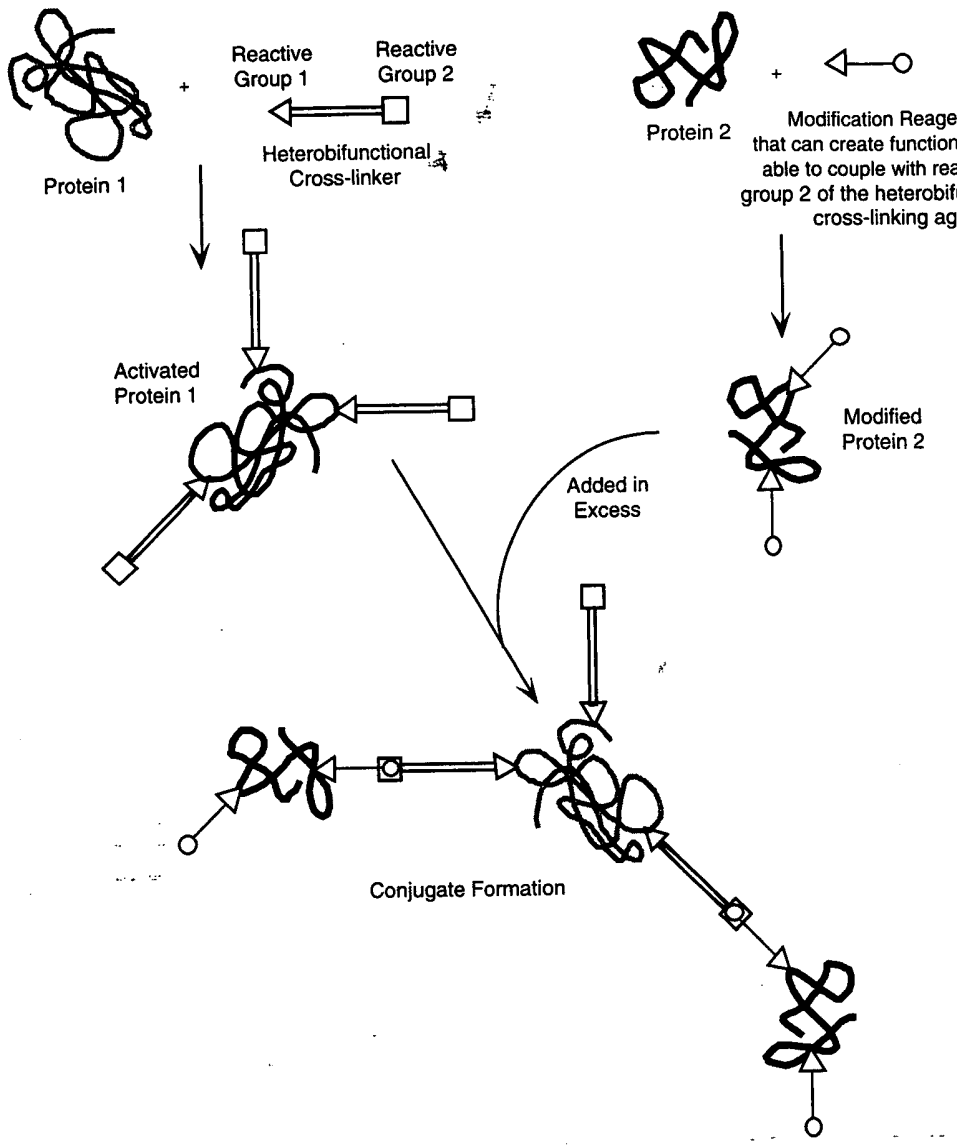


Figure 19 Heterobifunctional cross-linking agents used in multistep protocols result in the best control over the products formed.

single step to multistep. Likewise, control of the chemistry of conjugation increases as the reagent systems evolve from simple homobifunctional to site-directed heterobifunctional. It may appear to be a paradox, but as the method of conjugation gets more complex the result is less potential for side reactions and therefore fewer products being formed. Therefore, multistep processes using advanced heterobifunctional reagents are the best combination to ensure that the protein conjugate formed is indeed the one desired.

2. Modification of Sugars, Polysaccharides, and Glycoconjugates

The basic units of food energy for cells and living organisms consist of polysaccharide or simple sugars, principally glucose and its derivatives. Biological molecules themselves often contain carbohydrate or are made exclusively of such components. Complex carbohydrate "trees" frequently project off the surface of cells, providing specific points of attachment or sites of recognition. Lipids and proteins that contain these components may possess them to give identity or partial hydrophilicity to their parent structures.

Many of the macromolecules that are the subject of modification or conjugation reactions contain significant proportions of carbohydrate. Reactions can be designed to target directly these polysaccharide portions, either selectively modifying them with small, detectable compounds or using them as conjugation bridges to couple with other macromolecules. The reactivity of carbohydrate molecules in such derivatizations is an important factor in the success of many bioconjugate techniques.

This section describes the basic chemical attributes of carbohydrate molecules. Principle sites of reactivity on carbohydrates are discussed with the aim of developing a rational approach to using them in modification and conjugation procedures.

2.1. Carbohydrate Structure and Function

Carbohydrates are characterized by the presence of polyhydroxylic aldehyde or polyhydroxylic ketone structures or polymers of such units. Sugars and polysaccharides have definite three-dimensional structures that are important for many biological functions. They are hydrophilic and thus easily accessible to aqueous reaction mediums. The chemistry of bioconjugation using carbohydrate molecules begins with an understanding of the building blocks of polysaccharide molecules.

Basic Sugar Structure

The simplest carbohydrate, called a monosaccharide, is composed of a structure that can not be hydrolyzed to simpler polydroxylic compounds. A disaccharide is a carbohydrate that contains two of these basic units, and a polysaccharide contains many polyhydroxylic monomers.

A monosaccharide that contains an aldehyde group is called an aldose, and one that contains a ketone group is a ketose. Monosaccharides are further classified by the number of carbon atoms they contain. Thus, a five-carbon sugar is known as a pentose and a six-carbon sugar, a hexose. All monosaccharides, by virtue of their aldehyde or ketone functional groups, are reducing sugars—that is, they are able to reduce Fehling's or Tollen's reagent.

The aldehyde or ketone group of monosaccharides can undergo an intramolecular reaction with one of its own hydroxyl groups to form a cyclic, hemiacetal or hemiketal structure, respectively (Fig. 20). In aqueous solutions, this cyclic structure actually predominates. The open-chain aldehyde or ketone form of monosaccharides is in equilibrium with the cyclic form, but the open structure exists less than 0.5% of the time in aqueous environments. It is the open form that reduces Fehling's or Tollen's

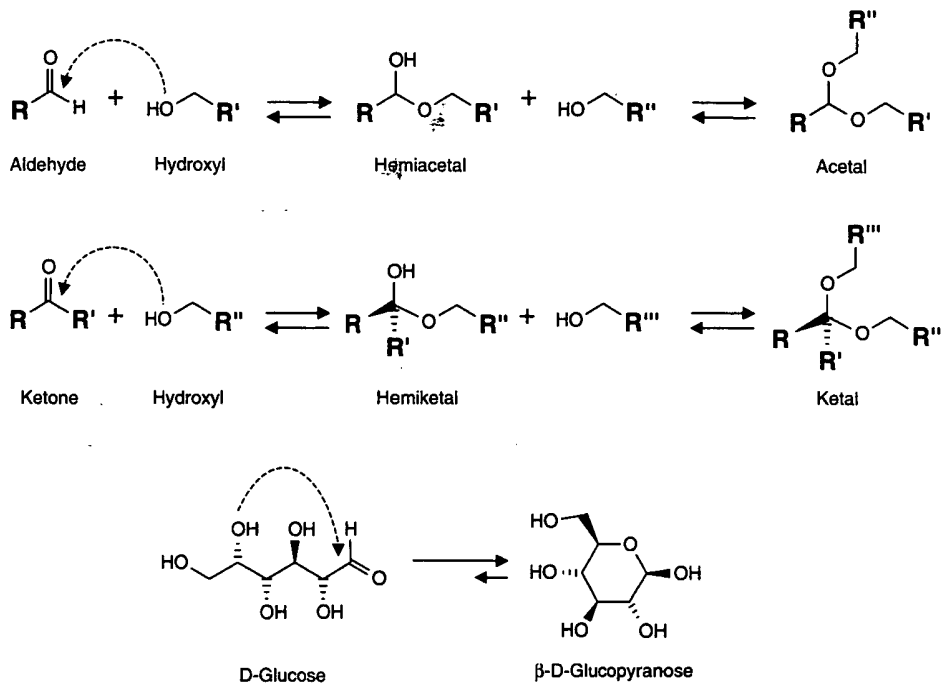


Figure 20 Carbonyl groups and hydroxyls may react to form acetal or ketal products. Sugars naturally undergo these reactions to form ring structures in aqueous solution.

reagent. However, due to this predominance of the cyclic structure of monosaccharides, they do not have the capability of reacting with bisulfite or Schiff reagent as do normal unblocked aldehydes and ketones. Thus, the carbonyl functional groups of sugars have reduced reactivity, because of hemiacetal and hemiketal formation.

Figure 21 shows the structures of some of the most common monosaccharide molecules: D-glyceraldehyde, D-erythrose, D-ribose, D-arabinose, D-xylose, D-glucose, D-glucosamine, *N*-acetyl-D-glucosamine, D-mannose, D-galactose, D-galactosamine, *N*-acetyl-D-galactosamine of the aldose family and dihydroxyacetone, D-ribulose, D-fructose, D-*N*-acetylneuraminic acid of the ketose family. Formation of the cyclic structure of each of these sugars can result in one of two stereoisomers, designated α and β, depending on the orientation of the aldehyde group or ketone group during hemiacetal formation. For aldoses, the α form is drawn in the standard Haworth projection with the number 1 carbon hydroxyl pointing down. For ketoses, the α form consists of the No. 2 carbon hydroxyl pointing down. All the common monosaccharide structures shown in Fig. 21 are in the β-stereoisomer form.

Since in aqueous solutions the cyclic form of monosaccharides is in equilibrium with their corresponding open forms, the α and β structures continually interconvert. At equilibrium, one form usually predominates. For instance, glucose dissolved in water consists of about a 2:1 ratio of β-D-glucose to α-D-glucose. Although their chemical constituents are identical, the biochemical properties between the α and the β forms can be quite different. Monosaccharides linked together to form disaccharides and polysaccharides cannot continue to interconvert and are therefore frozen in the α or β forms. Changing one monosaccharide in a complex carbohydrate to its opposite

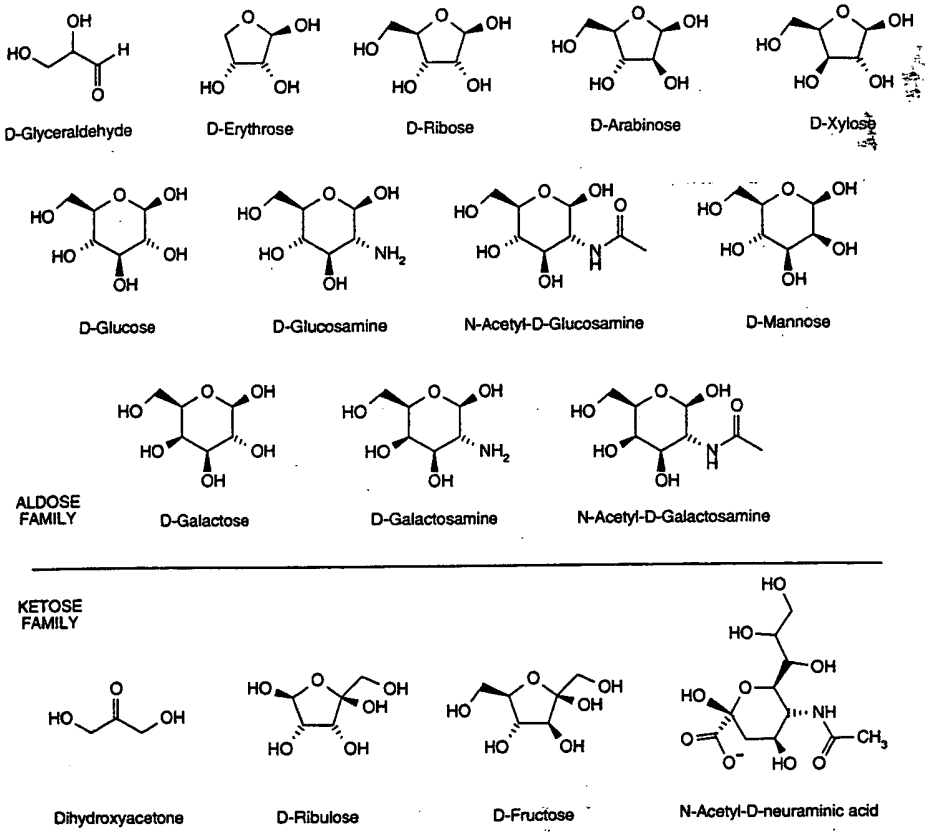


Figure 21 Common monosaccharides of the aldose and ketose families found in biological molecules.

stereoisomer form can produce radical structural changes in the polysaccharide chain and significantly alter its biochemical properties.

Sugar Functional Groups

Monosaccharide functional groups consist of either a ketone or an aldehyde, several hydroxyls, and the possibility of amine, carboxylate, sulfate, or phosphate groups as additional constituents. Amine-containing sugars may possess a free primary amine, but often are modified to the *N*-acetyl derivative, such as the *N*-acetylglucosamine residue of chitin. Sulfate-containing monosaccharides frequently are found in certain mucopolysaccharides, including chondroitin sulfate, dermatan sulfate, heparin sulfate, and keratin sulfate (Fig. 22). Carboxylate-containing sugars include sialic acid as well as many aldonic, uronic, oxoaldonic, and ascorbic acid derivatives (Fig. 23). Phosphate-containing monosaccharides are almost exclusively created in metabolic processes involving energy utilization, such as in the production of glucose 1-phosphate formed during glycogen breakdown and glucose 6-phosphate produced during glycolysis. Perhaps the most common phosphate sugar derivative, however, is the 5′-phosphate of D-ribose or D-2-deoxyribose found as a repeating component of RNA and DNA, respectively.

Modification and conjugation reactions can be designed to target many of these

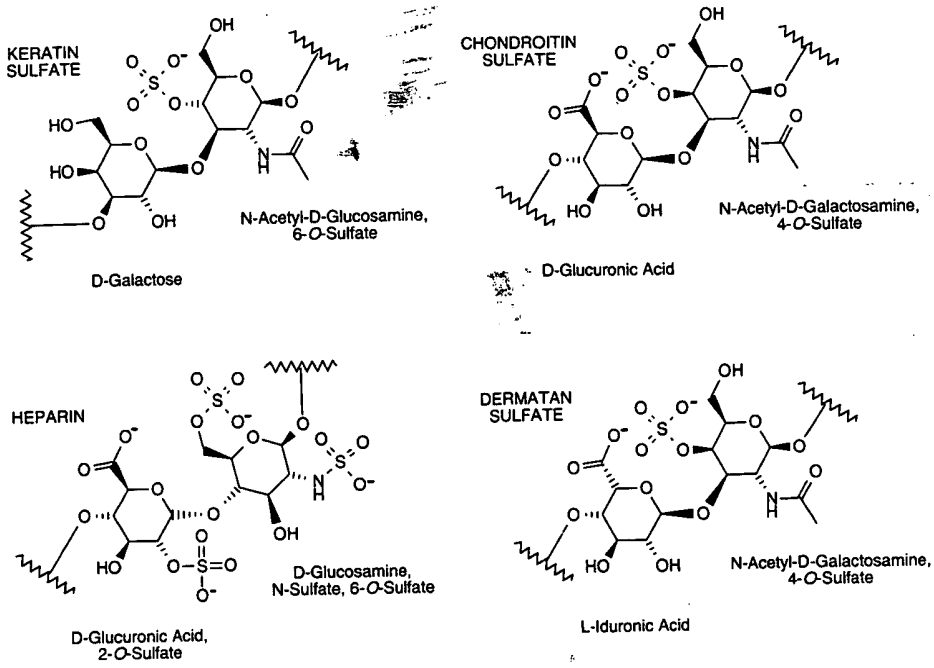


Figure 22 Common sulfonated polysaccharides of biological origin.

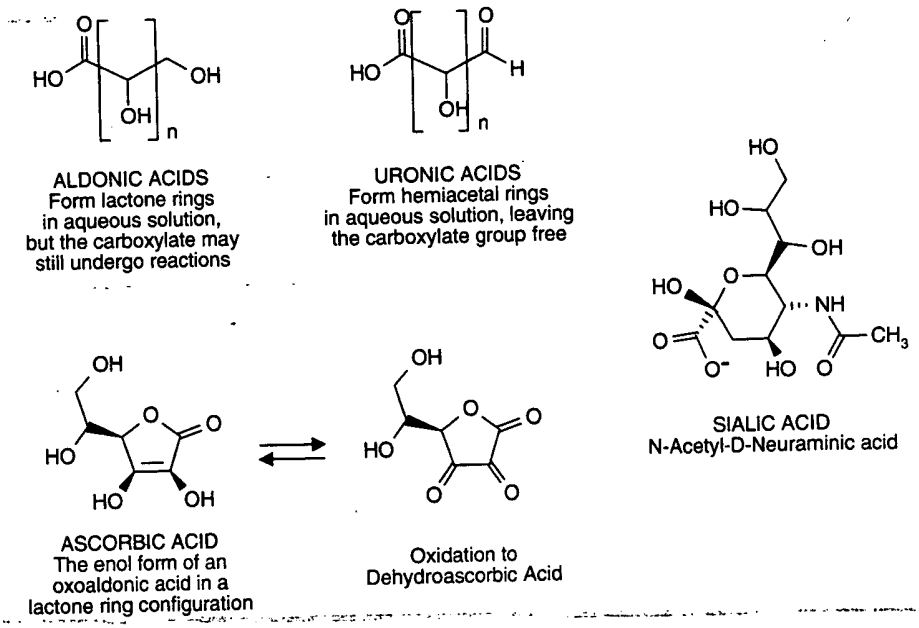


Figure 23 Monosaccharides containing carboxylate groups. Sialic acid often is found at the termina residues of polysaccharides within glycoproteins.

functional groups. Sugar hydroxyl groups, for example, may be derivatized by acylating or alkylating reagents, similar to the principal reactions of primary amines (Section 1). However, acylation of a hydroxyl group usually creates an unstable ester derivative that is subject to hydrolysis in aqueous solution. An exception to this is acylation by a carbonylating reagent such as CDI (Chapter 2, Section 4.2) or DSC (Chapter 2, Section 4.3), which can produce stable carbamate linkages after subsequent conjugation with an amine containing molecule. By contrast, alkylating reagents, such as alkyl halogen compounds (Chapter 2, Section 4.6) typically form more stable ether bonds after reaction with hydroxyls. Figure 24 shows the reactions associated with alkylation and acylation of hydroxyl residues.

Carbohydrates containing hydroxyl groups on adjacent carbon atoms may be treated with sodium periodate (Section 4.4) to cleave the associated carbon-carbon bond and oxidize the hydroxyls to reactive formyl groups (Bobbitt, 1956). Modulating the concentration of sodium periodate can direct this oxidation to modify exclusively sialic acid groups (using 1 m*M* concentration) or to convert all available diols to aldehydes (using 10 m*M* or greater concentrations). Specific monosaccharide residues may be targeted with selective sugar oxidases to generate similar aldehyde functions only on discrete points of a polysaccharide chain (Section 4.4) (Avigad *et al.*, 1962; Gahmberg, 1978). The creation of formyl groups in this manner may be done on purified polysaccharide molecules, as in the case of soluble dextrans (Chapter 15, Section 2.1), or may be selectively performed on carbohydrate constituents of glycoproteins and other glycoconjugates. Once formed, aldehyde groups may be covalently coupled with amine-containing molecules by reductive amination using sodium cyanoborohydride (Chapter 3, Section 4) (Dottavio-Martin and Ravel, 1978; Cabacungan *et al.*, 1982).

The native reducing ends of carbohydrates also may be conjugated to amine-containing molecules by reductive amination. The reaction, however, typically is less efficient than using periodate-created aldehydes, since the open structure is in low concentration in aqueous solutions compared to the cyclic hemiacetal form. The

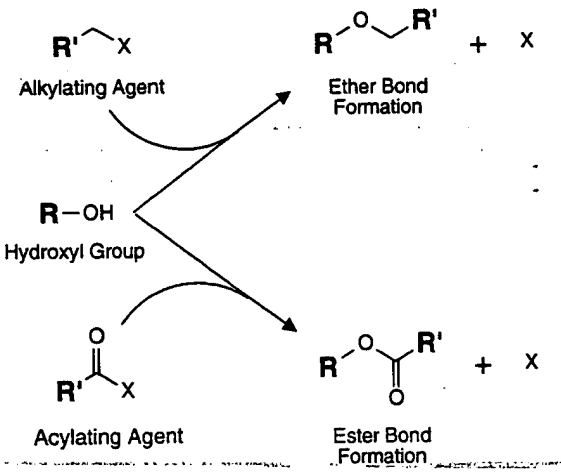


Figure 24 Hydroxyl groups within sugar residues may undergo alkylation or acylation reactions, forming ether or ester linkages.

reaction is usually allowed to continue for a week or more to reach good yields of coupling. Proteins may be modified to contain carbohydrate using this procedure (Gray, 1974, 1978; Baues and Gray, 1977; Schwartz and Gray, 1977).

The reducing ends of oligosaccharides can be modified with β-(*p*-aminophenyl)ethylamine to yield terminal arylamine derivatives (Zopf *et al.*, 1978a; Jeffrey *et al.*, 1975). The aromatic armines then can be diazotized for coupling to active-hydrogen-containing molecules, such as the tyrosine phenolic residues in proteins (Zopf *et al.*, 1978b). Alternatively, the arylamines may be transformed into isothiocyanate derivatives for coupling to amine-containing molecules, such as proteins (D. F. Smith *et al.*, 1978). The aromatic amine also may be used to conjugate the modified oligosaccharide directly with amine-reactive cross-linking agents or probes.

Another potential reaction of created or native aldehyde groups on carbohydrates is with hydrazide functionalities to form hydrazone linkages. Hydrazide-containing probes or cross-linking reagents may be conjugated with periodate-oxidized polysaccharides or with the reducing ends of sugars. The hydrazone bonds may be reduced with sodium cyanoborohydride to more stable linkages (Chapter 2, Section 5.1). The reduction step is recommended for long-term stability of cross-linked molecules. An example of this modification strategy is the use of biotin-hydrazide (Chapter 8, Section 3.3) to label specifically glycoproteins at their carbohydrate locations.

Reducing sugars can be detected by reaction with phenylhydrazine to yield a hydrazone product, except that the result of the reaction is not what one might imagine giving the structure of aldoses and ketoses. Glucose, for example, can react with phenylhydrazine to yield the anticipated 1-phenylhydrazone derivative. In an excess of phenylhydrazine, however, the reaction continues to yield a 1,2-phenylhydrazone product, called an osazone, with concomitant production of aniline and ammonia (Fig. 25). Exactly how the No. 2 hydroxyl group gets oxidized to react with another molecule of phenylhydrazine is not entirely clear, but it probably proceeds through an enol intermediate. This reaction is typical of all α-hydroxy aldehydes and α-hydroxy ketones, not just those occurring in carbohydrate molecules. Thus, glucose, mannose, and fructose all yield the same osazone product upon reaction with phenylhydrazine, since the stereochemical differences about carbons 1 and 2 are eliminated. Reversal of the phenylhydrazone linkage with an excess of benzaldehyde yields an osone, a 1-aldehyde-2-keto- derivative of the sugar. Many simple hydrazide-containing reagents probably are capable of forming similar 1,2-hydrazone derivatives with reducing sugars, provided their size does not cause steric difficulties.

Polysaccharides, glycoproteins, and other glycoconjugates therefore may be specifically labeled on their carbohydrate by creating aldehyde functional groups and subsequently derivatizing them with another molecule containing an amine or a hydrazide group. This route of derivatization is probably the most common way of modifying carbohydrates.

The hydroxyl residues of polysaccharides also may be activated by certain compounds that form intermediate reactive derivatives containing good leaving groups for nucleophilic substitution. Reaction of these activated hydroxyls with nucleophiles such as amines results in stable covalent bonds between the carbohydrate and the amine-containing molecule. Activating agents that can be employed for this purpose include carbonyl diimidazole (Chapter 2, Section 4.2 and Chapter 3, Section 3), certain chloroformate derivatives (Chapter 2, Section 4.3), tresyl- and tosyl chloride, cyanogen bromide, divinylsulfone, cyanuric chloride (Chapter 15, Section 1.1), disuccinimidyl carbonate (Chapter 4, Section 1.7), and various *bis*-epoxide compounds

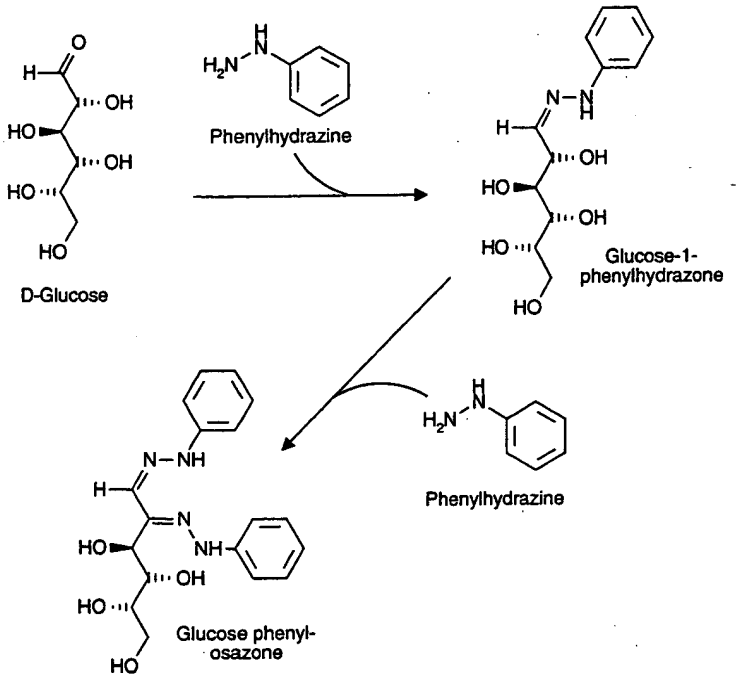


Figure 25 Phenylhydrazine can react with aldehyde or ketone groups within carbohydrates to give detectable products.

(Chapter 2, Section 1.7). Such activation steps are frequently done in nonaqueous solutions (i.e., dry dioxane, acetone, DMF, or DMSO) to prevent hydrolysis of the active species. Although many pure polysaccharides can tolerate these organic environments, many biological glycoconjugates cannot. Thus, these methods are suitable for activating pure polysaccharides such as dextran, cellulose, agarose, and other carbohydrates, but are not appropriate for modifying sugar residues on glycoproteins. Many of these hydroxyl-activating reagents also can be used to activate polysaccharide chromatography supports and other hydroxyl-containing synthetic polymers such as polyethylene glycol. For a complete treatment of polysaccharide chromatographic support activation through hydroxyl groups, see Hermanson *et al.* (1992). For a description of the activation of soluble polysaccharides and synthetic polymers, see Chapter 15.

The hydroxyl groups of carbohydrate molecules are only mildly nucleophilic—approximately equal to water in their relative nucleophilicity. Since the majority of reactive functional groups on bioconjugation reagents are dependent on nucleophilic reactions to initiate covalent bond formation, specific hydroxyl group modification is usually not possible in aqueous solution. Hydrolysis of the active groups on cross-linking reagents occurs faster than hydroxyl group modification, due to the relative high abundance of water molecules compared to the amount of carbohydrate hydroxyls present. In some cases, even if modification does occur, the resultant bond may be unstable. For instance, NHS esters (Chapter 2, Section 1.4) can react with hydroxyls to form ester linkages, which are themselves unstable to hydrolysis.

Anhydrides, such as acetic anhydride (Sections 4.2 and 5.1), may react with car-

bohydrate hydroxyls even in aqueous environments to form acyl derivatives. The reaction, however, is reversible by incubation with hydroxylamine at pH 10–11.

Epoxide-containing reagents, such as the homobifunctional 1,4-(butanediol) diglycidyl ether (Chapter 4, Section 7.1), can react with polysaccharide hydroxyl groups to form stable ether bonds. Bis-epoxy compounds have been used to couple sugars and polysaccharides to insoluble matrices for affinity chromatography (Sundberg and Porath, 1974). The reaction of epoxides, however, is not specific for hydroxyl groups and will cross-react with amine and sulfhydryl functional groups, if present.

Hydroxyl groups on carbohydrates may be modified with chloroacetic acid to produce a carboxylate functional group for further conjugation purposes (Plotz and Rifai, 1982). In addition, indigenous carboxylate groups, such as those in sialic acid residues and aldonic or uronic acid-containing polysaccharides, may be targeted for modification using typical carboxylate modification reactions (Chapter 2, Section 3). However, when these polysaccharides are part of macromolecules containing other carboxylic acid groups such as glycoproteins, the targeting will not be specific for the carbohydrate alone. Pure polysaccharides containing carboxylate groups may be coupled to amine-containing molecules by use of the carbodiimide reaction (chapter 3, Section 1). The carboxylate is activated to an O-acylisourea intermediate which is in turn attacked by the amine compound. The result is the formation of a stable amide linkage with loss of one molecule of isourea.

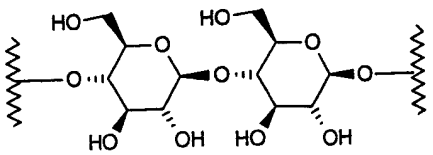
Carbohydrate molecules containing amine groups, such as D-glucosamine, may be easily conjugated to other macromolecules using a number of amine-reactive chemical reactions and cross-linkers (Chapter 2, Sections 1 and 2). Some polysaccharides containing acetylated amine residues, such as chitin which contains *N*-acetylglucosamine, may be deacetylated under alkaline conditions (Jeanloz, 1963) to free the amines (forming chitosan in this case).

Amine functional groups also may be created on polysaccharides (Section 4.3). The reducing ends of carbohydrate molecules (or generated aldehydes) may be reacted with small diamine compounds to yield short alkylamine spacers that can be used for subsequent conjugation reactions. Hydrazide groups may be similarly created using bis-hydrazide compounds (Section 4.5).

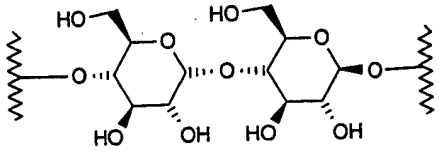
Phosphate-containing carbohydrates that are stable, such as the 5′-phosphate of the ribose derivatives of oligonucleotides, may be targeted for modification using a carbodiimide-facilitated reaction (Section 4.3). The water-soluble carbodiimide EDC can react with the phosphate groups to form highly reactive phospho-ester intermediates. These intermediates can react with amine- or hydrazide-containing molecules to form stable phosphoramidate bonds.

Polysaccharide and Glycoconjugate Structure

Aldose monosaccharide units are frequently bound together through the No. 1 carbon hydroxyl group of one sugar to another sugar's No. 4 or 6 hydroxyl group, forming a complete acetal linkage. Two monosaccharides coupled in this fashion are termed a disaccharide. Numerous monosaccharides bound together to form a chain are called a polysaccharide. The most abundant polysaccharides in nature, starch and cellulose, consist of glucose bound together in α-1,4, β-1,4, and, to a lesser extent, α-1,6 acetal linkages (Fig. 26). Although the hemiacetal, cyclic structure of individual sugars shows some reversibility under equilibrium conditions, the acetal linkage between two monosaccharides is quite stable, only hydrolyzing under severe pH extremes.



Cellobiose repeating unit
of cellulose;
D-Glucose joined in
β-1,4-linkages



Maltose repeating unit
of starch (amylose);
D-Glucose joined in
α-1,4-linkages

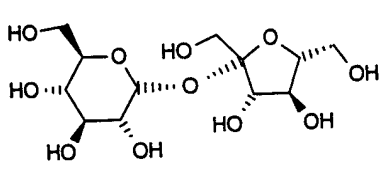
Figure 26 The repeating units of cellulose and starch, two of the most common polysaccharides in nature.

Similarly, ketose sugars participate in polysaccharide formation by reaction of their anomeric carbon with a hydroxyl of another monosaccharide to create a ketal linkage. The acetal and ketal bonds within polysaccharides are termed O-glycosidic linkages.

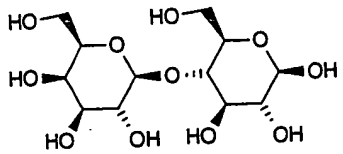
Hemiacetal hydroxyl groups of carbohydrate molecules also may be coupled to amine-containing molecules to form *N*-glycosidic linkages, such as those in nucleic acids and ogligonucleotides.

Polysaccharides may or may not have reducing power, depending on the way they are linked together and whether the terminal, potentially reducing end is available. The structure of simple disaccharides can illustrate this point. Of the most common disaccharides, sucrose and lactose, sucrose is a nonreducing sugar since β-D-fructose is linked through its reducing C-2 hydroxyl, and lactose remains a reducing sugar, since the terminal glucose is linked to β-D-galactose through its C-4 hydroxyl, leaving its reducing end free (Fig. 27).

Polysaccharide synthesis is under enzymatic control, but does not occur from a



Sucrose;
α-D-glucose and β-D-fructose
joined in an a-1,2 linkage;
A non-reducing sugar



Lactose;
β-D-galactose and β-D-glucose
joined in a β-1,4 linkage;
A reducing sugar

Figure 27 Comparison of a reducing and a nonreducing disaccharide.

template as in protein synthesis. For this reason, each molecule of a particular polysac charide will have its own unique molecular weight. The molecular weight of a carboh drate polymer is usually expressed as an average. Starch or cellulose chains, for exam ple, may vary by several hundred thousand in their molecular weights betwee individual molecules. For an excellent review of carbohydrate chemistry, see Binkle (1988).

Due to their polyhydroxylic structures, all carbohydrates are polar and will posses associated water molecules in aqueous solution, but they may not be fully wate soluble. Large polysaccharides such as cellulose form intricate matrices created fron extensive hydrogen bonding. Neighboring monosaccharide units hydrogen bon within the same chain, whereas neighboring polymers form interchain hydroge bonds between hydroxyls. The three-dimensional structure of a carbohydrate to large extent is determined by these hydrogen bonds—sometimes resulting in sheete or helical structures, as in the triple helix of agarose polysaccharide chains. Water wil be intimately associated in this internal arrangement, but the overall multipolyme structure often is too large to allow for complete water solubility. For a review, see Prei (1980).

Polysaccharide solubility in aqueous solutions usually is dependent on polymer siz and its allied three-dimensional structure. Even water-insoluble carbohydrates may b solubilized by controlled hydrolysis of O-glycosidic linkages to create smaller polysac charide molecules. Thus, cellulose may be solubilized by heating in an alkaline solu tion until the polymers are broken up sufficiently to reduce their average molecula weight. Many such soluble forms of common polysaccharides are available commer cially.

Carbohydrate also is an important constituent of many biological molecules. Poly saccharides may be found covalently conjugated to proteins and lipids, forming gly coproteins, proteoglycans, glycolipids, and lipopolysaccharides. Such glycoconju gates are produced in the cell through controlled, enzymatic processes. With proteins the modification occurs after translational synthesis of the polypeptide chain at th ribosome.

Proteins newly synthesized on ribosomes may be transported to the Golgi appara tus where specific glycosyl transferases catalyze the coupling of monosaccharides t the polypeptide chains. Glycoproteins and mucoproteins are formed by the couplin of polysaccharides through O-glycosidic linkages to serine, threonine, or hy droxylysine in addition to *N*-glycosidic linkages with the amide side chain group o asparagine (Fig. 28). For reviews of glycoconjugate structure and function, see Hyne (1987); Lennarz (1980); Jentoft (1990); and Steer and Ashwell (1986).

The structure of most glycoprotein carbohydrate consists of a complex, branche heteropolysaccharide with the sugars mannose, *N*-acetyl glucosamine, sialic acid galactose, and L-fucose being prevalent. Asparagine-linked polysaccharides are wel characterized and are known to be constructed of a core unit consisting of thre mannose residues and two *N*-acetyl glucosamine (GlcNAc) residues. The GlcNA residues are bound to the Asp side chain amide nitrogen through a β1 linkage (Korn field and Kornfield, 1985). The three mannose groups then usually form the firs branch point in the oligosaccharide chain (Fig. 29). Much of the detailed structura knowledge of glycoconjugates is developed using controlled chemical or enzymati degradation of the polysaccharides followed by analysis by gas chromatography-an mass spectrometry (Biermann and McGinnis, 1989; McCleary and Matheson, 1986 Sweeley and Nunez, 1985; Vliegenthart *et al.*, 1983).

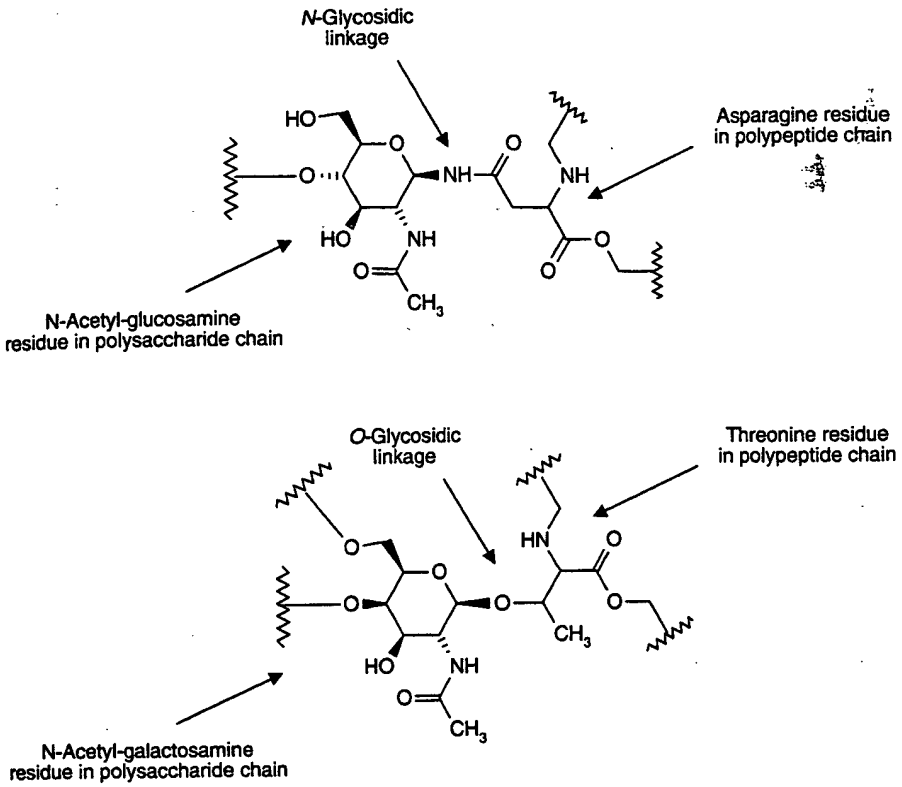


Figure 28 Common attachment points for polysaccharide chains on glycoproteins.

The content by weight of carbohydrate in glycoproteins may vary from only a few percent to as much as 70% in some proteins in mucous secretions. Although the exact function of the polysaccharide in most glycoproteins is unknown, in some cases it may provide hydrophilicity, recognition, and points of noncovalent interaction with other proteins through lectin-like affinity binding. In addition, extensive polysaccharide modification is helpful in preventing proteolytic digestion of the underlying polypeptide chain.

Another form of post-translational modification that may add carbohydrate to a polypeptide is nonenzymatic glycation. This reaction occurs between the reducing ends of sugar molecules and the amino groups of proteins and peptides. The aldehyde group of a reducing sugar first forms a reversible Schiff's base linkage with the α-amino or ε-amino groups of the protein. This bond then can undergo an Amadori rearrangement to form a stable ketoamine derivative (Fig. 30). The result is a blocked amine containing a sugar derivative with available hydroxyl residues. This reaction commonly occurs with proteins continually exposed to reducing sugars, such as glucose in blood. The measurement of glycated hemoglobin is a clinically important parameter in the management of diabetes mellitus. Increases in the blood sugar level in diabetes cause concomitant increases in the level of nonenzymatic glycation of blood proteins. Measuring the relative amount of glycated hemoglobin provides the physician with information concerning a diabetic patient's blood glucose control.

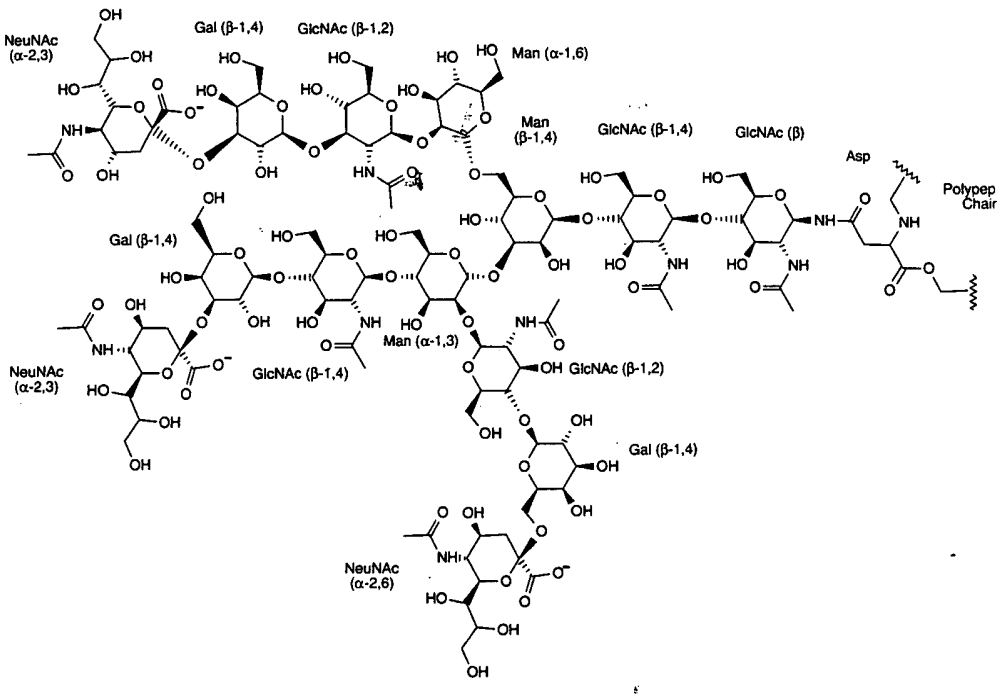


Figure 29 The complex structure of an asparagine-linked polysaccharide. Note the branched nature of the polymer with terminal sialic acid residues on each chain.

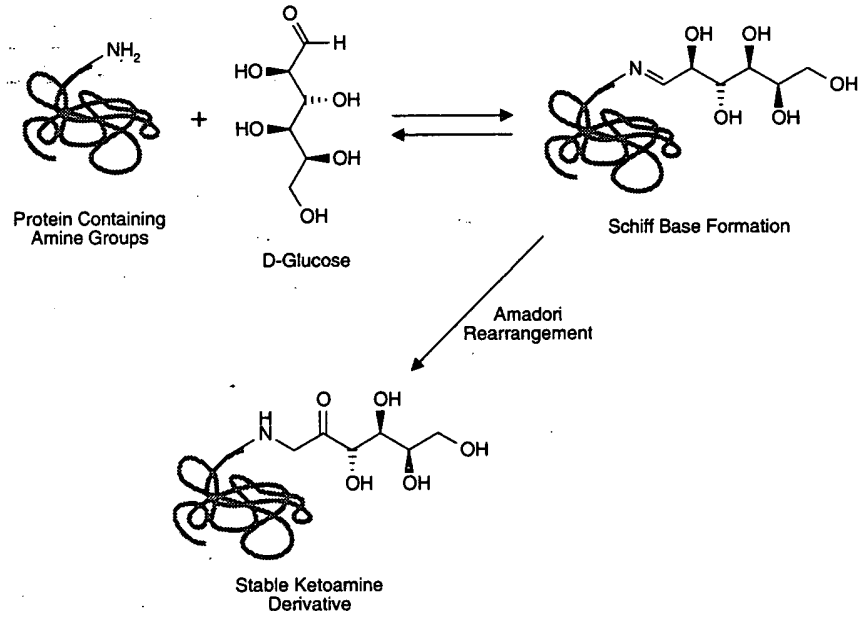


Figure 30 A reducing sugar may modify protein amine groups through Schiff base formation followed by an Amadori rearrangement to give a stable ketoamine product. Glucose is a common *in vivo* modifier of blood proteins through this process.

2.2 Carbohydrate Cross-linking Methods

The presence of carbohydrate on biomolecules provides important points of attachment for modification and conjugation reactions. Coupling only through polysaccharide chains often can direct the reaction away from active centers or critical points in protein molecules, thus preserving activity. Cross-linking strategies involving polysaccharides or glycoconjugates usually involve a two- or three-step reaction sequence. If no reactive functional groups other than hydroxyl groups are present on the carbohydrate, then the first step is to create sufficiently reactive groups to couple with the functional groups of a second molecule.

Perhaps the easiest way to target specifically polysaccharides on glycoproteins is through mild sodium periodate oxidation. Periodate cleaves adjacent hydroxyl groups in sugar residues to create highly reactive aldehyde functional groups (section 4.4). It is an aqueous reaction that is tolerated by most biological glycoconjugates and pure polysaccharide molecules. Particularly convenient is that the level of periodate addition can be adjusted to cleave selectively only certain sugars in the polysaccharide chain. A concentration of 1 m*M* sodium periodate specifically oxidizes sialic acid residues to aldehydes, leaving all other monosaccharides untouched. Increasing the concentration to 10 m*M*, however, will cause oxidation of other sugars in the carbohydrate chain, including galactose and mannose residues on glycoproteins. The generated aldehydes then can be used in coupling reactions with amine- or hydrazide-containing molecules to form covalent linkages. Amines can react with formyl groups under reductive amination conditions using a suitable reducing agent such as sodium cyanoborohydride. The result of this reaction is a stable secondary amine linkage (Chapter 2, Section 5.3). Hydrazides spontaneously react with aldehydes to form hydrazone linkages, although the addition of a reducing agent greatly increases the efficiency of the reaction and the stability of the bond (Chapter 2, Section 5.1).

Oxidized glycoconjugates usually are stable enough to be stored freeze-dried without loss of activity prior to a subsequent conjugation reaction. Storage in solution, however, may cause slow polymerization if the molecule also contains amine groups, as in glycoproteins. Sometimes the protein can be treated to block its amines prior to periodate oxidation, as in the procedure often used with the enzyme horseradish peroxidase (HRP) (Chapter 16, Section 1), thus eliminating the potential for self-conjugation.

If the second molecule to be coupled to the oxidized glycoconjugate already has the requisite amines or hydrazide groups, then directly mixing the two components together in the presence of a reductant is all that is needed. This is an example of a two-step procedure. However, if the second molecule possesses none of the appropriate functional groups for coupling, then modifying it to contain them must be done prior to the conjugation reaction (see Sections 4.3 and 4.5). Thus, a three-step protocol results. The use of other functional groups (either indigenous or created) on polysaccharide molecules to effect a cross-linking reaction can be done in similar two- or three-step strategies.

Occasionally, it is important to conjugate a polysaccharide-containing molecule to another molecule while retaining, as much as possible, the carbohydrate's original chemical and three-dimensional structure. For instance, in the preparation of immunogen conjugates by coupling a polysaccharide molecule to a carrier, care should be taken to preserve the structure of the carbohydrate to ensure antibody recognition of

the native molecule. In this case, periodate oxidative techniques may not be best choice to effect cross-linking due to the potential for extensive ring opening throughout the chain. Under controlled conditions, however, where periodate is carefully used in limiting quantities, this method has proved successful in creating oligosaccharide–carrier conjugates (Anderson *et al.*, 1989).

Retention of native carbohydrate structure also is important in applications that utilize the conjugated polysaccharide in binding studies with receptors or lectins. In these cases, the carbohydrate should be modified at limited sites, preferentially only at its reducing end.

3. Modification of Nucleic Acids and Oligonucleotides

The nucleic acid polymers DNA and RNA form the most basic units of information storage within cells. The conversion of their unique information code into proteins and enzymes is the fundamental step in controlling all cellular processes. Targeting segments of this encoded data with labeled probes that are able to bind to specific genetic regions allows detection, localization or quantification of discrete oligonucleotides. This targeting capability is made possible by the predictable nature of nucleic acid interactions. Despite the complexity of the genetic code, the base-pairing process, which causes one oligonucleotide to bind to its complementary sequence, is rather simplistic. Nucleic acids are the only type of complex biological molecule wherein their binding properties can be fully anticipated and incorporated into synthetic oligonucleotide probes. Thus, a short DNA segment can be synthetically designed and used to target and hybridize to a complementary DNA strand within a much larger chromosome. If the small oligonucleotide is labeled with a detectable component that does not interfere in the base-pairing process, then the targeted DNA can be assayed.

Bioconjugate techniques involving nucleic acids are becoming one of the most important application areas of cross-linking and modification chemistry. As the secrets of the genetic code are broken by such mammoth efforts as the Human Genome Project, knowledge of the DNA sequence that governs specific protein synthesis is leading to diagnostic tests able to assess the presence of critical genetic markers associated with certain disease states. To test for particular target sequences, complementary oligonucleotide probes are used that possess conjugated enzymes, fluorophores, haptens, radiolabels, or other such groups that can be used to detect a hybridization signal. Such oligonucleotide conjugates can be used to discover target sequences in blots, electrophoresis gels, tissues, or cells, or immobilized to surfaces or in solution.

The power and advantages of assessing cellular processes at their most fundamental level is propelling the science of oligonucleotide probe detection into the most prominent position in bioconjugate chemistry. Some are predicting that in the not-too-distant future hundreds of tests will be done routinely in the physician's office—each monitoring different aspects of genetic information—all with the use of specific oligonucleotide probes.

In this section, the chemistry and structure of nucleic acids and oligonucleotides is discussed with a view to creating functional conjugates with detectable molecules. The corresponding strategies and protocols associated with DNA or RNA modification and conjugation can be found in Chapter 17.

3.1. Polynucleotide Structure and Function

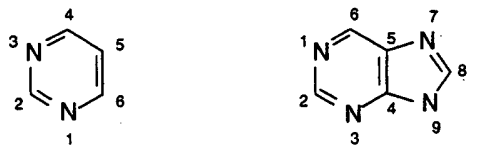
Polymers of nucleic acids are characterized by the types of base residues present and the structure of their sugar backbone. The bases are nitrogenous ring compounds consisting of either purine or pyrimidine derivatives. A purine is a fused-ring compound containing one six-member ring attached to a five-member ring, whereas a pyrimidine consists of a single six-member ring (Fig. 31).

Nucleic acids can contain of any one of three kinds of pyrimidine ring systems (uracil, cytosine, or thymine) or two types of purine derivatives (adenine or guanine). Adenine, guanine, thymine, and cytosine are the four main base substituents found in DNA. In RNA molecules, three of these four bases are present, but with thymine replaced by uracil to make up the fourth. Some additional minor derivatives are found in messenger RNA (mRNA), transfer RNA (tRNA), and ribosomal RNA (rRNA), particularly the N^4, N^4-dimethyladenine and N^7-methylguanine varieties.

Nucleic acid sugar residues are attached to the associated base units in an *N*-glycosidic bond, involving the No. 1 nitrogen of pyrimidine bases or the No. 9 nitrogen of purines directly linked to the No. 1 carbon of the monosaccharide derivative (Fig. 32). The sugar group consists of either a β-D-ribose unit (found in RNA) or a β-D-2-deoxyribose unit (in DNA) (Fig. 33). In mRNA and rRNA, a minor sugar derivative, a 2′-*O*-methylribosyl group, also is found.

The nomenclature of nucleic acid chemistry further characterizes the structure of the associated groups. A *nucleoside* contains only a base group and an attached sugar. A *nucelotide* consists of a base and a sugar plus a phosphate group. At this point, the naming system gets somewhat confusing due to the fact that the nucleoside name is a derivative of the base name. Table 2 shows this relationship and their associated abbreviations (which are simpler to remember).

In each nucleotide monomer of DNA or RNA molecules, a phosphate group is attached to the C-5 hydroxyl of each sugar residue in an ester (anhydride) linkage. These phosphate groups in turn are linked in diester bonds to neighboring sugar groups of adjacent nucleotides through their 3′-ribosyl hydroxyl to create the oligonucleotide polymer backbone (Fig. 34). Thus, the phosphate–sugar repeating unit produces the linear sequence within the DNA or RNA structure, while the four types of base units protrude out from this backbone, creating the unique code making up the genetic information.



Pyrimidine;
1,3-Diazine

Purine;
7*H*-Imidazo[4,5-*d*]pyrimidine

Figure 31 The pyrimidine and purine ring structures common to nucleic acids.

Table 2 Nucleic Acid Nomenclature

Base name	Nucleoside name[a] (base + sugar)	Nucleotide name[b] (base + sugar + phosphate)
Adenine	Adenosine	Adenosine monophosphate (AMP)
Guanine	Guanosine	Guanosine monophosphate (GMP)
Cytosine	Cytidine	Cytidine monophosphate (CMP)
Thymine	Thymidine	Thymidine monophosphate (TMP)
Uracil	Uridine	Uridine monophosphate (UMP)

[a]For deoxyribose nucleosides, add "deoxy" before the nucleoside name. For example, adenosine becomes deoxyadenosine.

[b]For the presence of two phosphate groups, the names are changed to diphosphate. For three phosphate groups, the terminology is triphosphate.

group on the No. 5 carbon of thymine. Cytosine, by contrast, contains an additional site of unsaturation between carbons 3 and 4 as well as an amine group on C-4 instead of a ketone (Fig. 35).

Figure 36 indicates major sites of reactivity within the ring structures for nucleophilic displacement reactions. Cytosine, thymine, and uracil all react toward nucleophilic attack at the same two sites, the C-4 and C-6 positions. The presence of powerful nucleophiles, even at neutral pH, can lead to significant base modification or cleavage with pyrimidine residues (Debye, 1947). For instance, hydrazine spontaneously adds to the 5,6 double bond, initiating further ring reactions, which eventually leads to oligonucleotide degradation. A similarly strong nucleophile, hydroxylamine, is almost entirely specific for modifying pyrimidines. It too can add to the 5,6 double bond, creating a 6-hydroxylamino derivative. In general, the pyrimidines can undergo reactions at the 5,6 double bond leading to a stable modification at the C-5 position (Fig. 37).

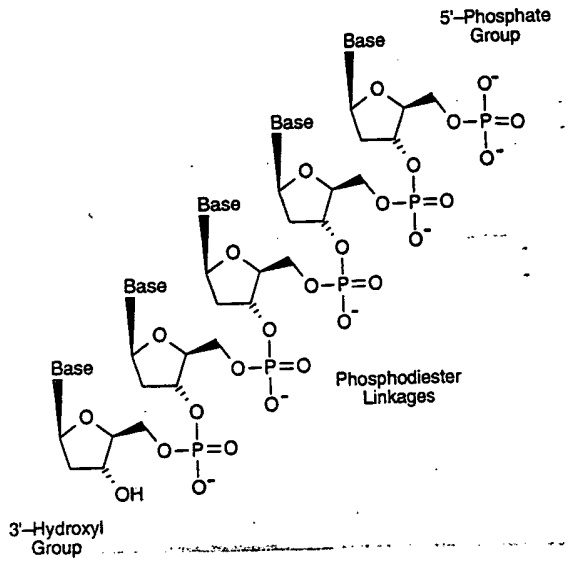


Figure 34 Polynucleotides are formed through phosphodiester bonds linking the associated sugar groups together. In DNA, the 3′-hydroxyl of one deoxyribose unit is bound to the 5′-hydroxyl of the next, creating direction in the polymer backbone.

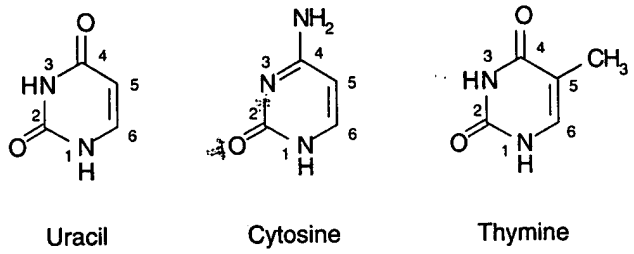


Figure 35 The three pyrimidine bases common to nucleic acid construction. Cytosine and thymine are found in DNA, while in RNA, uracil residues replace thymine. The associated sugar groups are bound in *N*-glycosidic linkages to the N-1 nitrogen.

Addition of a nucleophile to the C-6 position of cytosine often results in fascile displacement reactions occurring at the N_4 location. With hydroxylamine attack, nucleophilic displacement causes the formation of an N_4-hydroxy derivative. A particularly important reaction for bioconjugate chemistry, however, is that of nucleophilic bisulfite addition to the C-6 position. Sulfonation of cytosine can lead to two distinct reaction products. At acid pH wherein the N-3 nitrogen is protonated, bisulfite reaction results in the 6-sulfonate product followed by spontaneous hydrolysis. Raising the pH to alkaline conditions causes effective formation of uracil. If bisulfite addition is done in the presence of a nucleophile, such as a primary amine or hydrazide compound, then transamination at the N_4 position can take place instead of hydrolysis (Fig. 38). This is an important mechanism for adding spacer arm functionalities and other small molecules to cytosine-containing oligonucleotides (see Chapter 17, Section 2.1).

Electrophilic reagents also can modify the pyrimidine rings of nucleic acids. Alkylation and acylation reactions can take place at several sites on all three bases. Figure 39 illustrates the principal locations where electrophilic attack can occur. In particular, the heteroatoms (oxygen and nitrogen) are the best positions of high electron density, therefore functioning as nucleophiles in reaction processes. Of the pyrimidine residues, however, it is the N-3 position of cytosine derivatives that is the most susceptible to alkylation. Reactions can occur with ethylenimine compounds (Section 4.3), alkyl halogens (Chapter 2, Section 2.1), epoxides (Chapter 2, Section 1.7), and many other strong alkylating agents [for review, see Brown (1974)].

Acylation reactions can be done at the nucleophilic sites on pyrimidines using

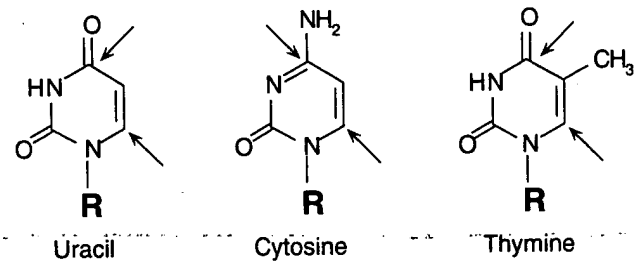


Figure 36 Pyrimidine bases are subject to nucleophilic displacement reactions primarily at the C-4 and C-6 positions.

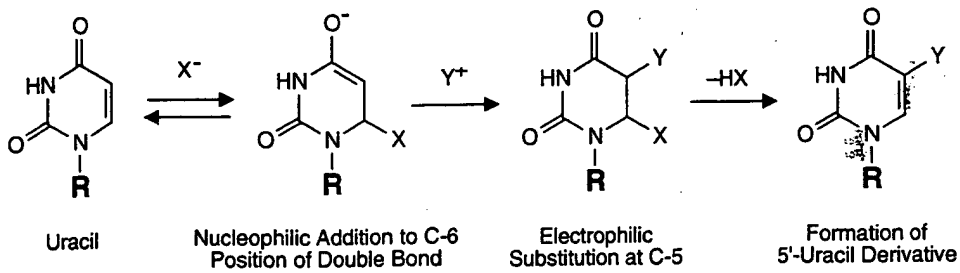


Figure 37 Nucleophilic addition at C-6 of the pyrimidine double bond can cause electrophilic substitution to occur at the C-5 position.

activated forms of carboxylic acids. Acylation of functional groups in nucleotides typically is used for protection during synthesis (Reese, 1973). However, for bioconjugate applications, the reactivity of native groups on pyrimidines is not as great as that obtained using an amine-terminal spacer derivative, such as those described in Chapter 17, Section 2.1. Yields and reaction rates are typically low for direct acylation or alkylation of pyrimidine bases, especially in aqueous environments.

The N-3 position of uracil also can be modified with carbodiimide reagents. In

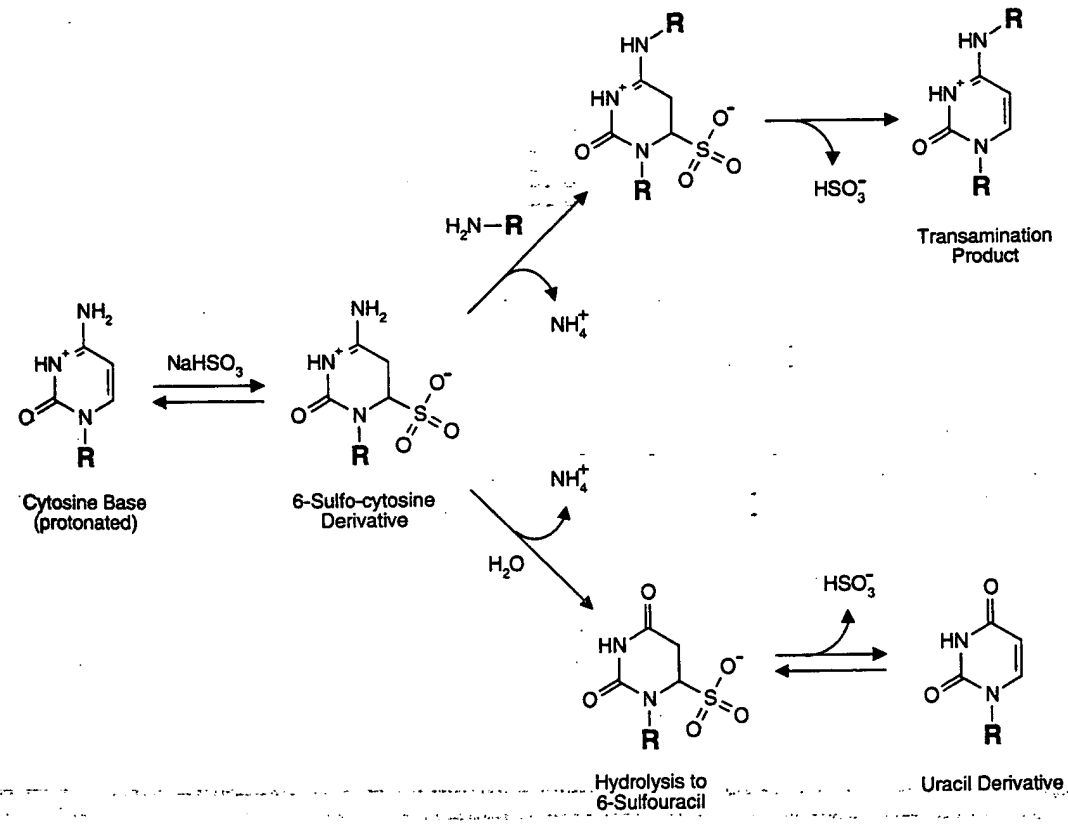


Figure 38 Reaction of bisulfite with cytosine bases is an important route of derivatization. It can lead to uracil formation or, in the presence of an amine (or hydrazide)-containing compound, transamination can occur, resulting in covalent modification.

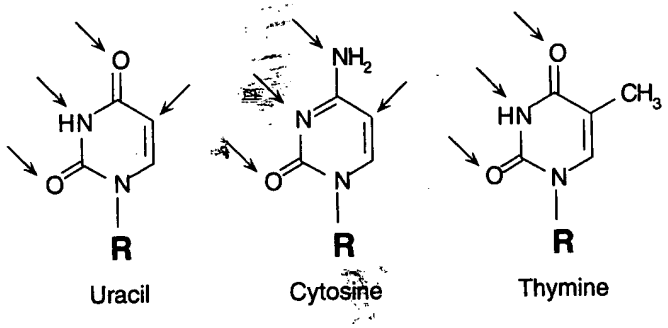


Figure 39 Potential sites of electrophilic attack on pyrimidine bases.

particular, the water-soluble carbodiimide CMC [1-cyclohexyl-3-(2-morpholino ethyl) carbodiimide, as the metho *p*-toluene sulfonate salt] can react with the N-3 nitrogen at pH 8 to give an unstable, charged adduct. The derivative is reversible at pH 10.5, regenerating the original nucleic acid base (Fig. 40). Cytosine is unreactive in this process.

Halogenation of pyrimidine bases may be done with bromine or iodine. Bromination occurs at the C-5 of cytosine, yielding a reactive derivative that can be used to couple diamine spacer molecules by nucleophilic substitution (Fig. 41) (Traincard *et al.*, 1983; Sakamoto *et al.*, 1987; Keller *et al.*, 1988). Other pyrimidine derivatives also are reactive to bromine compounds at the C-5 position. Either an aqueous solution of bromine or the compound *N*- bromosuccinimide can be used for this reaction. The brominated derivatives can be used to couple amine-containing compounds to the pyrimidine ring structure (chapter 17, Section 2.1).

Other reactions characterized for pyrimidine residues include mercuration at C-5 of cytosine or uracil (Hopman *et al.*, 1986), cyloaddition to the 5,6 double bond of thymine and uracil (Cimino *et al.*, 1985), and thiolation at the C-4 amino group of cytosine (Malcolm and Nicolas, 1984).

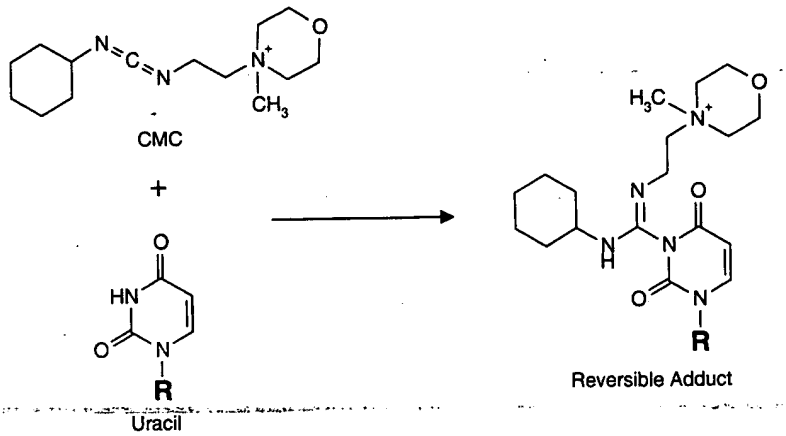


Figure 40 The carbodiimide CMC can react with the N-3 nitrogen to yield a reversible product.

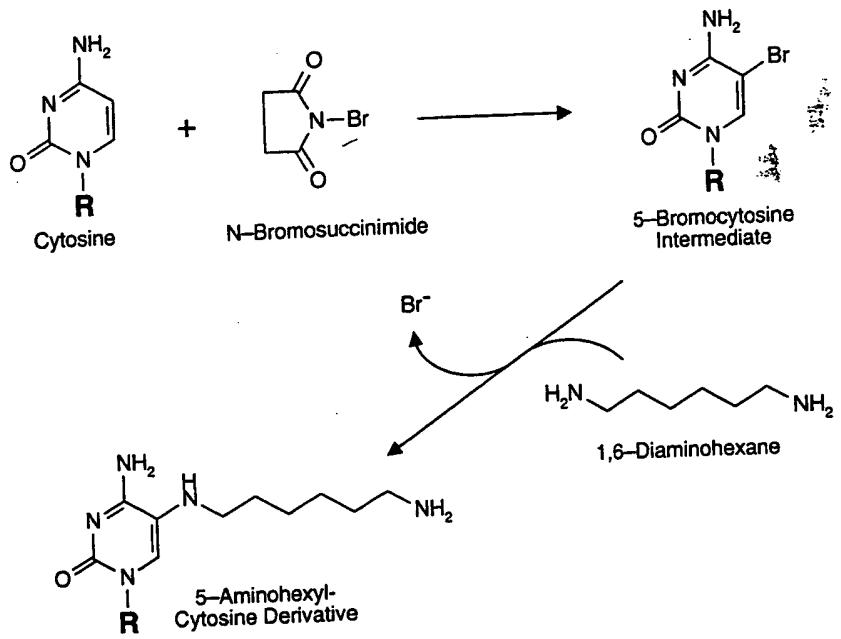


Figure 41 Cytosine bases are susceptible to bromination at the C-5 double bond position, resulting in active intermediates capable of reacting with amine nucleophiles.

Adenine and Guanine Residues

The purine bases of nucleic acids are constructed of a two-ring system made from a pyrimidine-type, six-member ring fused with a five-member imidazole ring. Adenine and guanine are present in both RNA and DNA. They differ in their six-member ring structures by an additional point of unsaturation between C-6 and N-1 (in adenine) and by the presence of amine or ketone groups attached to C-2 or C-6 (Fig. 42). Attachment to ribose or deoxyribose in nucleosides is made through an *N*-glycosidic linkage at N-9 of the imidazole ring on either purine.

As in the case of pyrimidine bases discussed previously, adenine and guanine are subject to nucleophilic displacement reactions at particular sites on their ring structures (Fig. 43). Both compounds are reactive with nucleophiles at C-2, C-6, and C-8, with C-8 being the most common target for modification. However, the purines are

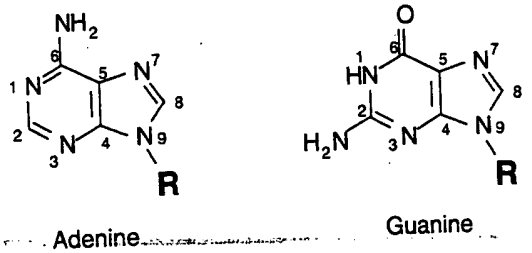
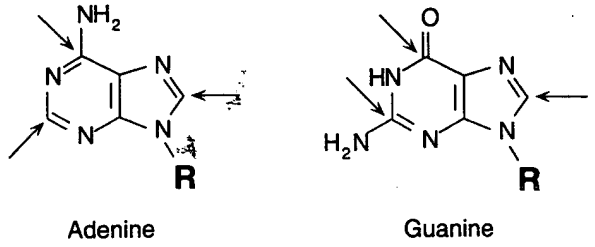


Figure 42 The structures of the common purine bases of RNA and DNA. The associated sugar groups are bound in *N*-glycosidic linkages to the N-9 position.



Adenine Guanine

Figure 43 Primary nucleophilic displacement sites on purine bases.

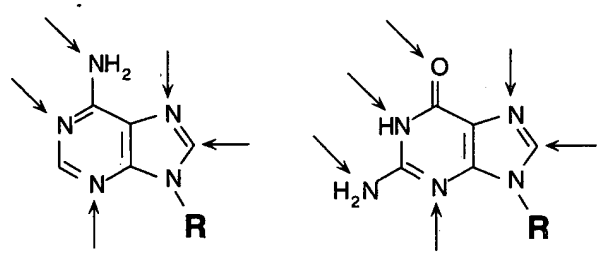
much less reactive to nucleophiles than the pyrimidines. Hydrazine, hydroxylamine, and bisulfite—all important reactive species with cytosine, thymine, and uracil—are almost unreactive with guanine and adenine.

With purines, reaction with electrophilic species is the most important route to derivatization. Figure 44 identifies the major sites of electrophilic attack on adenine and guanine. On both bases it is the heteroatoms that make up the majority of sites. Alkylation reactions thus can occur at N-1, N-3, and N-7 in adenine or N-3 and N-7 in guanine. However, the greatest location of electron density (nucleophilicity) occurs at N-7 on the imidazole ring of guanine, followed by N-1 of adenine. According to Brown (1974), the order of reactivity of nucleosides toward alkylation by esters of strong acids is guanine > adenosine > cytidine >> uridine (nearly unreactive).

As with pyrimidines, the water-soluble carbodiimide CMC may react with guanine derivatives to give a reversible adduct at N-1 (Fig. 45). Raising the pH to highly alkaline conditions regenerates the purine group. Adenine residues, however, display no reactivity in this process.

One of the most important reactions of purines is the bromination of guanine or adenine at the C-8 position. It is the site that is the most common point of modification for biconjugate techniques using purine bases (Fig. 46). Either an aqueous solution of bromine or the compound *N*-bromosuccinimide can be used for this reaction. The brominated derivatives then can be used to couple amine-containing compounds to the pyrimidine ring structure by nucleophilic substitution (Chapter 17, Section 2.1).

Adenine also may undergo an additional reaction at its C-6 amine group using a Fischer–Dimroth rearrangement mechanism. Alkylation at N-1 can result in a rearrangement to give the C-6 alkylated product. The reaction at N-1 usually requires



Adenine Guanine

Figure 44 Electrophilic attack can occur at a number of sites on both purine bases.

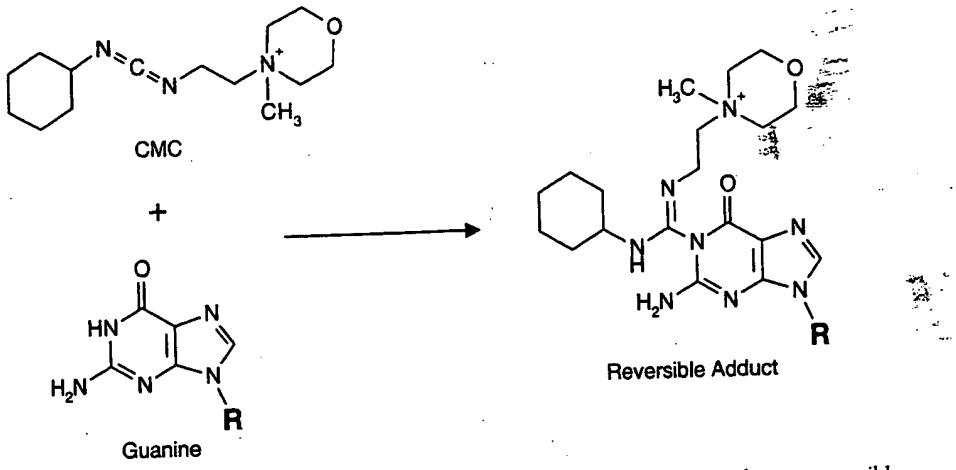


Figure 45 The carbodiimide CMC can react with guanine at the N-1 position to form a reversible complex.

extended time to obtain good yields. For instance, alkylation with iodoacetic acid takes 5–10 days at pH 6.5. Under alkaline conditions and elevated temperatures, the six-member ring then is broken and reformed, resulting the 6-aminoalkylated product containing a terminal carboxylate group (Fig. 47). The resultant acid can be used in further derivatization reactions to facilitate conjugate formation (Lowe, 1979).

An additional reaction reported for adenine involves the coupling of glutaraldehyde to the 6-amino group (Matthews and Kricka, 1988). However, reaction at this group with electrophilic reagents such as those discussed in Part II proceeds more slowly than

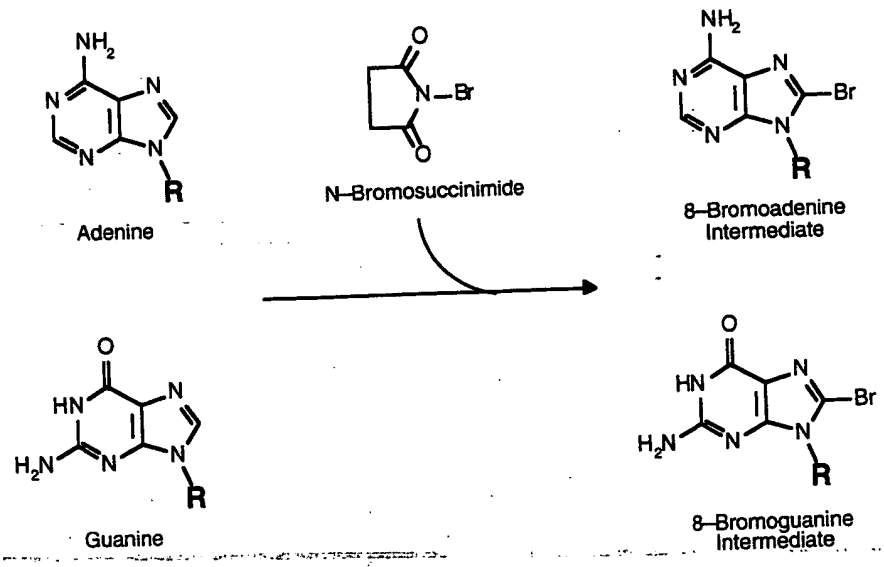


Figure 46 The purine bases are subject to bromination reactions at the C-8 position, forming an important reactive intermediate for derivatization purposes.

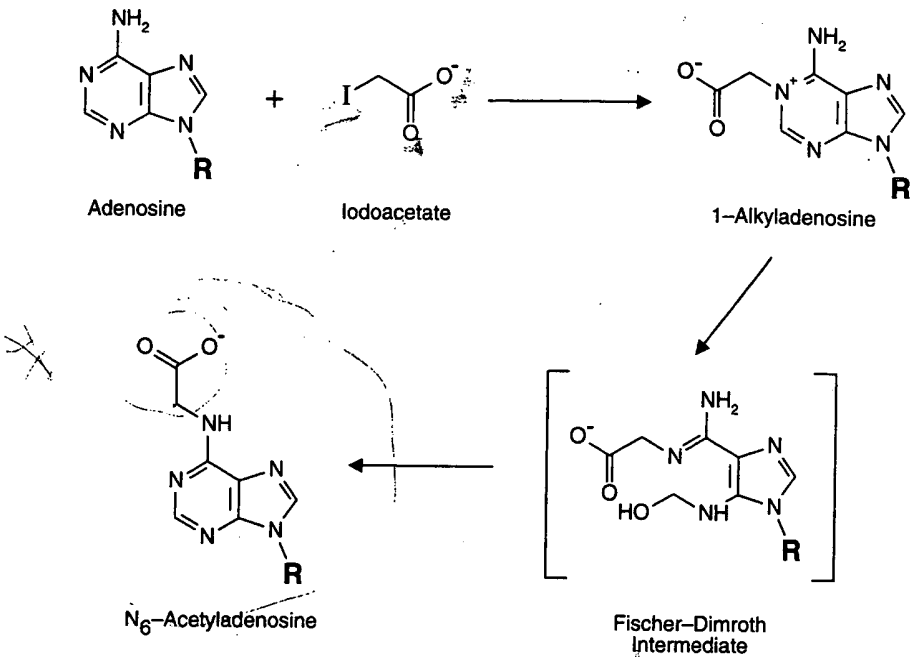


Figure 47 Alkylation reactions can occur at the N-1 position of adenosine, resulting in a Fischer–Dimroth rearrangement to yield an N_6 derivative.

that possible using a primary aliphatic amine. In general, bioconjugate chemistry done with nucleic acid bases involves the formation of an intermediate derivative containing a spacer arm terminating in an amine, sulfhydryl, or carboxylate to obtain acceptable reactivity and yields.

Sugar Groups

The sugar portion of oligonucleotides is a 5-carbon pentose occurring in one of two forms. In RNA, it is β-D-ribose in a ring structure. In DNA, the monosaccharide is β-D-2-deoxyribose, wherein the number 2′ carbon of the ring lacks an hydroxyl group. An individual nucleotide will have its 1′ hydroxyl group of the ribose unit tied up in an *N*-glycosidic bond with the associated base and its C-5 hydroxyl group bound to phosphate in an ester linkage. If the nucleotide is of the deoxy form, then the only remaining hydroxyl is on the 3′ carbon of the sugar unit. Ribonucleic acids, by contrast, contain a diol group formed from the two hydroxyls on the 2′ and 3′ carbons of ribose (Fig. 48). Polymers of nucleic acids are created through diester phosphate bonds, mainly connected between the 5′ hydroxyl of one sugar group and the 3′ hydroxyl of the next adjacent sugar. Thus, DNA contains no hydroxyl groups except the single one at the 3′ terminal of each strand. RNA has one hydroxyl at each nucleotide sugar unit and a diol group at the 3′ end.

Conjugation or modification reactions may be done through the 3′ hydroxyl group of deoxyribonucleic acids or the 2′,3′-diol of ribonucleic acids. Hydroxyls may be targeted for coupling using strong alkylating agents under alkaline conditions. Epox-

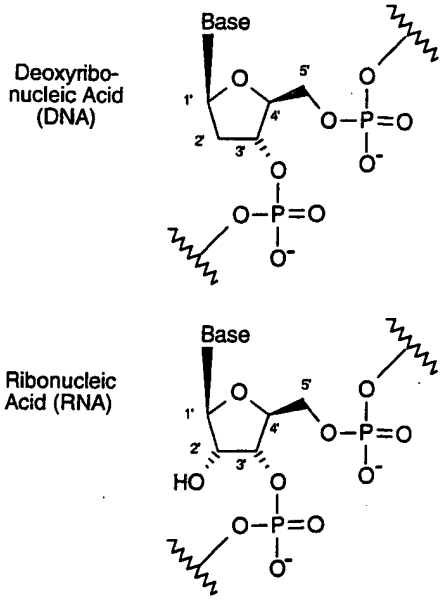


Figure 48 The similar structures of DNA and RNA basic units.

ide compounds (Chapter 4, Section 7) are particularly effective at modifying hydroxyl groups. The most common method of conjugation through nucleotide sugar units, however, is periodate oxidation of the adjacent hydroxyls of ribonucleic acids. Treatment with periodate breaks the carbon—carbon bond between the two hydroxyl residues and creates two aldehyde groups (Seela and Waldeck, 1975). A procedure for oxidizing carbohydrates with sodium periodate can be found in Section 4.4. This method can be used to create RNA conjugates through directed coupling only at the 3′ end or to immobilize ribonucleic acids such as ATP to insoluble supports for affinity chromatography (Lowe, 1979).

Phosphate Groups

The phosphate groups of nucleotides are joined to the 5′ hydroxyl group of the sugar component in an ester or anhydride linkage. Several forms of nucleoside phosphate compounds are possible, containing up to three esterified phosphate groups polymerized off the ribose or deoxyribose unit. The presence of these groups contributes an overall negative charge to the nucleotide—minus two for the terminal phosphate group and minus one for each internal phosphate under alkaline conditions. Multiple esterified phosphates contain considerable potential energy from their easily hydrolyzed anhydride bonds. This energy is the basis for many biochemical transformations in biological systems. It is the triphosphate form of nucleosides that is utilized in DNA and RNA synthesis *in vivo*. However, nucleoside triphosphates and diphosphates such as ATP and ADP have numerous contributions to cellular metabolism beyond just oligonucleotide construction. Controlled hydrolysis of their multiple phosphate ester bonds releases energy for many biological operations. Other derivatives of nucleoside phosphate compounds provide cofactors for enzymes (such as coenzyme-A) or are

involved in signal transduction processes [such as cyclic AMP (cAMP)]. Figure 49 shows some of these common nucleoside phosphate derivatives.

The phosphate groups of nucleotides may be targeted for modification reactions using condensation agents such as carbodiimides. In aqueous environments, EDC (Chapter 3, Section 1.1) may be used to couple amine-containing compounds to the terminal phosphate group of an oligonucleotide, forming a phosphoramidate linkage. In DNA or RNA chains, the internal phosphate groups do not react under the pH conditions of the modification. In this way, the 5′ phosphate group may be specifically targeted for modification or conjugation, thus avoiding potential interference with hydrogen bonding interactions with complementary polynucleotide strands. Chapter 17, Sections 2.1 and 2.2 describe the use of this reaction in bioconjugate applications.

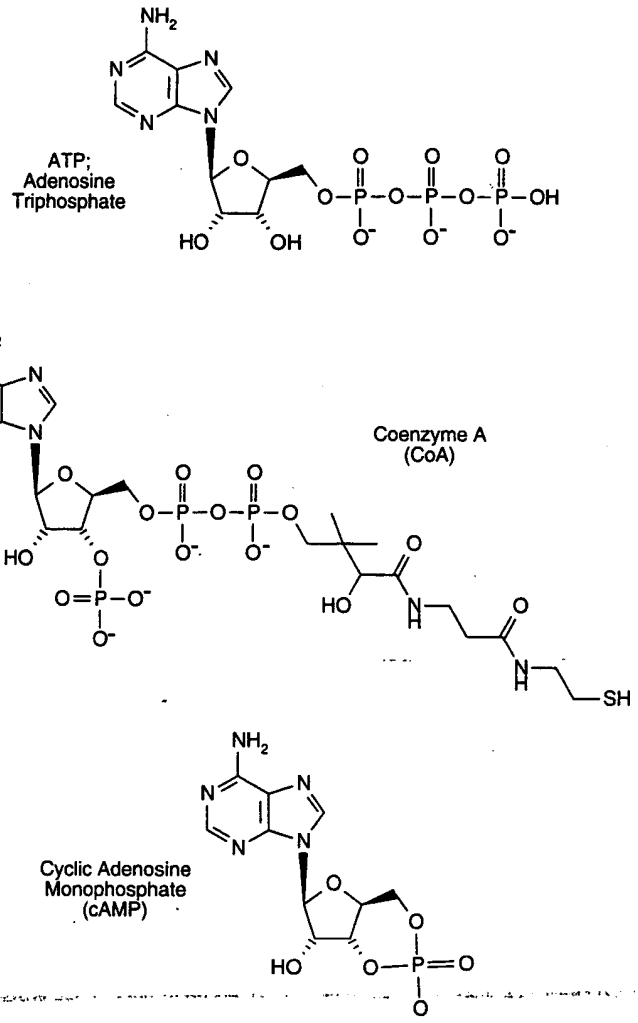


Figure 49 Nucleotide derivatives have additional functions *in vivo* beyond their role in oligonucleotide construction.

Another phosphate modification procedure that is effective at adding detectable components to oligonucleotide probes is to use a phosphoramidite derivative. The common method of automated oligonucleotide synthesis is to use phosphoramidite chemistry to add nucleotides to the growing sequence. A functionalized phosphoramidite nucleotide derivative can be added at particular points in the synthetic process to create labeled probes of known structure. Nonnucleotide phosphoramidites also may be used to produce modified probes containing fluorescent molecules, biotin, chelating groups, or spacer groups with amines for further derivatization. Most of these techniques require an automated DNA synthesizer. The methods of DNA modification during synthesis have been reviewed and are beyond the scope of this book (Beaucage and Iyer, 1993).

RNA and DNA Structure

The nucleotides forming RNA or DNA molecules are linked together in phosphodiester bonds with sugar–phosphate repeating units. The esters are directionally linked between the 3′ hydroxyl of one ribosyl group and the 5′ hydroxyl of the next. The fundamental step in cellular DNA synthesis involves the reaction of a deoxynucleoside triphosphate group with the 3′ end of an existing chain. The nucleotide sequence of a new strand is enzymatically controlled by use of a complementary chain as a template. Each new nucleotide addition is facilitated by the energy released through hydrolysis of two phosphates from the triphosphate group of the incoming nucleoside. The resulting succession of nucelotides encodes the message for protein synthesis, with each three-base code signaling a particular amino acid in a polypeptide sequence.

Nucelotide bases projecting from the sugar–phosphate backbone of a polynucleotide are able to interact with other strands through hydrogen bonding. Hydrogen bonding can occur between cytosine and guanine base units in different strands of DNA through interaction of the C-2 ketone oxygen, the N-3 nitrogen, and C-4 amine groups of cytosine with the C-2 amine, N-1 nitrogen, and the C-6 ketone oxygen of guanine. In a similar fashion, thymine (or uracil) residues can hydrogen bond with adenine groups through the N-3 nitrogen and C-4 ketone oxygen of thymine interacting with the N-1 nitrogen and C-6 amine of adenine (Fig. 50).

This specific base-pairing capability of oligonucleotides defines the structure of

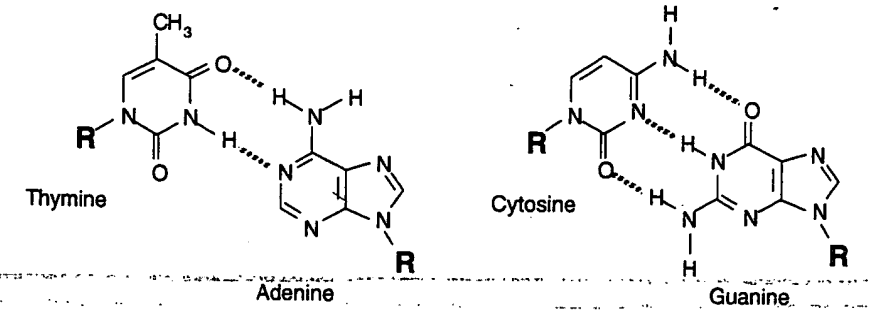


Figure 50 Base-pairing can occur between complementary bases in opposing oligonucleotide strands. These predictable interactions form the basis for using synthetic oligonucleotide probes to target particular DNA sequences.

complementary DNA molecules. In the classic Watson–Crick model, two complementary DNA strands interact in an antiparallel fashion to form a right-handed double helix. Thus, one chain runs in the 3′ to 5′ direction while the complementary chain runs in the 5′ to 3′ direction through the helical structure. This standard double helix, now called the B form, occurs often in aqueous solution and is the most stable structure under physiological conditions (Fig. 51). However, there are several other forms that double-stranded DNA can take in solution. Another right-handed helical construction, the A form, can occur under nonaqueous conditions and is more compact than the B form. A completely different DNA structure, the Z form, is a left-handed helix that can occur in some segments containing an abundance of alternating pyrimidines and purines. Short segments of Z structure have been found in some cells. Finally, some rare DNA sequences can form triple-helical regions through normal and non-Watson–Crick base-pairing.

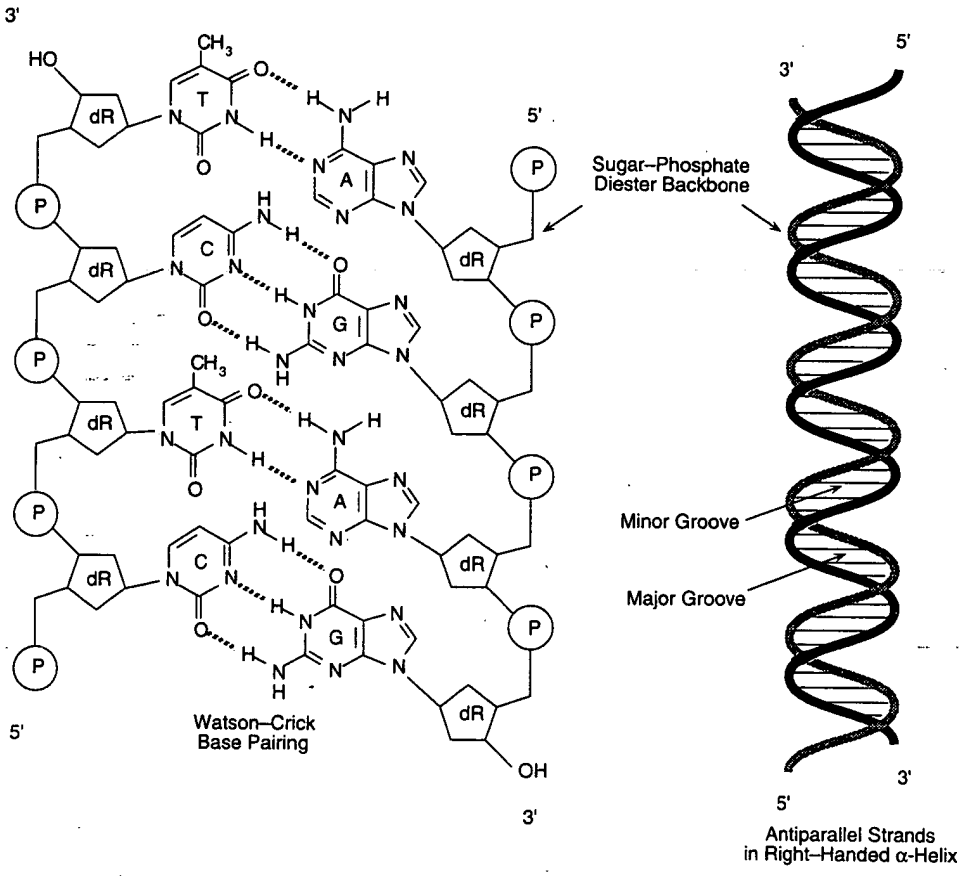


Figure 51 The classic Watson–Crick DNA double helix is formed through base-pairing interactions between two antiparallel strands. In physiological conditions, the two strands take on an α-helical shape with about 10 bp per turn of the helix. The phosphate–sugar backbone of the helix faces outward, while hydrogen bonding between opposing bases occurs in the middle of the wrapped strands. This configuration creates minor and major grooves between the phosphate–sugar backbones, potentially exposing the internal bases to interactions with other molecules.

Unlike the double-stranded nature of DNA, RNA molecules usually occur as single strands. This does not mean they are unable to base-pair as DNA can. Complementary regions within an RNA molecule often base-pair and form complex tertiary structures, even approaching the three-dimensional nature of proteins. Some RNA molecules, such as transfer RNA (tRNA) possess several helical areas and loops as the strand interacts with itself in complementary sections. Other hybrid molecules such as the enzyme RNase P contain protein and RNA portions. The RNA part is highly complex with many circles, loops, and helical regions creating a convoluted structure.

The predictable nature of DNA and RNA base-pairing make their interactions the most defined of any biological system. The specific affinity of one strand for its complementary sequence makes it possible to target genetic markers with extreme accuracy. Synthetic segments of RNA or DNA can be used to detect or quantify their complementary targets, even in highly dilute environments containing many other oligonucleotide molecules. If the oligonucleotide probe is labeled with a highly detectable component, then specific base-pairing interactions can be assayed. This ability has created an extensive utilization of labeled probes in molecular biology. Detection of target DNA or RNA can be done in cells, tissue sections, blots, or electrophoresis gels, or after amplification by PCR techniques, or in solution. The ability to detect single-copy genes through the use of labeled oligonucleotide probes will make this field one of the leading application areas for bioconjugate techniques.

3.2. Polynucleotide Cross-linking Methods

The unique properties of oligonucleotides create cross-linking options that are far different from those of any other biological molecule. Nucleic acids are the only major class of macromolecule that can be specifically synthesized *in vitro* by enzymatic means. The addition of modified nucleoside triphosphates to an existing DNA strand by the action of polymerases or transferases allows addition of spacer arms or detection components at random or discrete sites along the chain. Alternatively, chemical methods that modify nucleotides at selected functional groups can be used to produce spacer arm derivatives or activated intermediates for subsequent coupling to other molecules.

Thus, both chemical and enzymatic derivatization techniques can be used to form oligonucleotide probes of high activity in hybridization assays. The main consideration for successful polynucleotide cross-linking, as in other bioconjugate applications, is to avoid probe inactivation during the modification or conjugation process. Since the purpose in constructing a DNA or RNA probe is to hybridize and detect a complementary oligonucleotide through hydrogen bond interactions, any derivatization procedure that significantly interferes with Watson–Crick base-pairing should be avoided. This means that large amounts of base derivatization along a polynucleotide chain has potential for causing obstructions in the hybridization process, sometimes dramatically reducing or eliminating base-pairing efficiency. In general, base modifications within an oligonucleotide probe should be limited to no more than about 30–40 sites per 1000 bases to maintain hybridization ability.

By contrast, derivatization at the ends of an oligo or at the sugar–phosphate backbone usually produces little interference in base-pairing. Conjugates may be created by enzymatic polymerization of functionalized nucleoside triphosphates off the 3′ end or

by chemical modification of the 5′ phosphate group with minimal to no interference in hybridization potential. The application of these strategies to creating labeled oligonucleotide probes is discussed in Chapter 17.

4. Creating Specific Functional Groups

It is often desirable to alter the native structure of a macromolecule to provide functional targets for modification or conjugation. The use of most reagent systems requires the presence of particular chemical groups to effect coupling. For instance, heterobifunctional cross-linkers may contain two different reactive species that are directed against different functional groups. One target molecule must contain chemical groups able to react with one end of the cross-linker, while the other target molecule must contain groups able to react with the other end. Occasionally, the required chemical groups are not present on one of the target molecules and must be created. This usually can be done by reacting an existing chemical group with a modification reagent that contains or produces the desired functional group upon coupling. Thus, an amine can be "changed" into a sulfhydryl or a carboxylate can be altered to yield an amine simply by using the appropriate reagent.

This same type of modification strategy also can be used to create highly reactive groups from functional groups of rather low reactivity. For instance, carbohydrate chains on glycoproteins can be modified with sodium periodate to transform their rather unreactive hydroxyl groups into highly reactive aldehydes. Similarly, cystine or disulfide residues in proteins can be selectively reduced to form active sulfhydryls, or 5′ phosphate groups of DNA can be transformed to yield modifiable amines.

Alternatively, spacer arms can be introduced into a macromolecule to extend a reactive group away from its surface. The extra length of a spacer can provide less steric hindrance to conjugation and often yields more active complexes.

The use of modification reagents to create specific functional groups is an important technique to master. In one sense, the process is like using building blocks to construct on a target molecule any desired functional groups necessary for reactivity. The success of many conjugation schemes depends on the presence of the correct chemical groups. Care should be taken in choosing a modification strategy, however, since some chemical changes will radically affect the native structure and activity of a macromolecule. A protein may lose its capacity to bind a specific ligand. An enzyme may lose the ability to act upon its substrate. A DNA probe may no longer be able to hybridize to its complementary target. In many cases, the potential for inactivation relates to changing conformational structures, blocking active sites, or modifying critical functional groups. Trial and error and careful literature searches are often necessary to optimize any modification tactic.

4.1. Introduction of Sulfhydryl Residues (Thiolation)

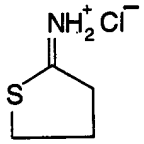
The sulfhydryl group is a popular target in many modification strategies. Cross-linking agents that have more than one reactive group often employ a sulfhydryl-reactive functional group at one end to direct the conjugation reaction to a particular part of a target macromolecule. The frequency of sulfhydryl occurrence in proteins or

other molecules is usually low (or nonexistent) compared to that of other groups like amines or carboxylates. The use of sulfhydryl-reactive chemical reactions thus can restrict modification to only a limited number of sites within a target molecule. Limiting modification greatly increases the chances of retaining activity after conjugation, especially in sensitive proteins like some enzymes. Unfortunately, sulfhydryl groups often need to be generated (from reduction of indigenous disulfides) or created (from use of the appropriate thiolation reagent systems). The following sections describe the most popular techniques of creating these functional groups. Some of these reagent systems are specifically designed to form —SH groups, while others are cross-linkers that also can serve the dual purpose of sulfhydryl-generating agents.

Sulfhydryl groups are susceptible to oxidation and formation of disulfide cross-links. To prevent disulfide bond formation, remove oxygen from all buffers by degassing under vacuum and bubbling an inert gas (i.e., nitrogen) through the solution. In addition, EDTA (0.01–0.1 *M*) may be added to buffers to chelate metal ions, preventing metal-catalyzed oxidation of sulfhydryls. Some proteins of serum origin (particularly BSA) contain so many contaminating metal ions (presumably iron from hemolyzed blood) that 0.1 *M* EDTA is required to prevent this type of oxidation.

Modification of Amines with 2-Iminothiolane (Traut's Reagent)

Perham and Thomas (1971) originally prepared an imidoester compound containing a sulfhydryl group, methyl 3-mercaptopropionimidate hydrochloride. The imidoester group can react with amines to form a stable, charged linkage (Chapter 2, Section 1.10), while leaving a sulfhydryl group available for further coupling (Fig. 52). Traut *et al.* (1973) subsequently synthesized an analogous reagent containing one additional carbon, methyl 4-mercaptobutyrimidate. Later, this compound was found to cyclize as a result of the sulfhydryl group reacting with the intrachain imidoester, forming 2-iminothiolane (Jue *et al.*, 1978). The cyclic imidothioester still can react with primary amines in a ring-opening reaction that regenerates the free sulfhydryl (Fig. 53).



Traut's Reagent;
2-Iminothiolane
MW 137.6
8.1 Å

Traut's reagent is fully water-soluble and reacts with primary amines in the pH range 7–10. The cyclic imidothioester is stable to hydrolysis at acid pH values, but its half-life in solution decreases as the pH increases beyond neutrality. However, even at pH 8 in 25 m*M* triethanolamine the rate of sulfhydryl formation without added primary amine was found to be negligible. On addition of dipeptide amine, the reagent reacted quickly as evidenced by the production of Ellman's reagent color. The rate of reaction also can be followed by 2-iminothiolane's absorbance at 248 nm (λ_{max}; $\varepsilon = 8840\ M^{-1}cm^{-1}$). As the cyclic imidate reacts with amines, its absorbance at

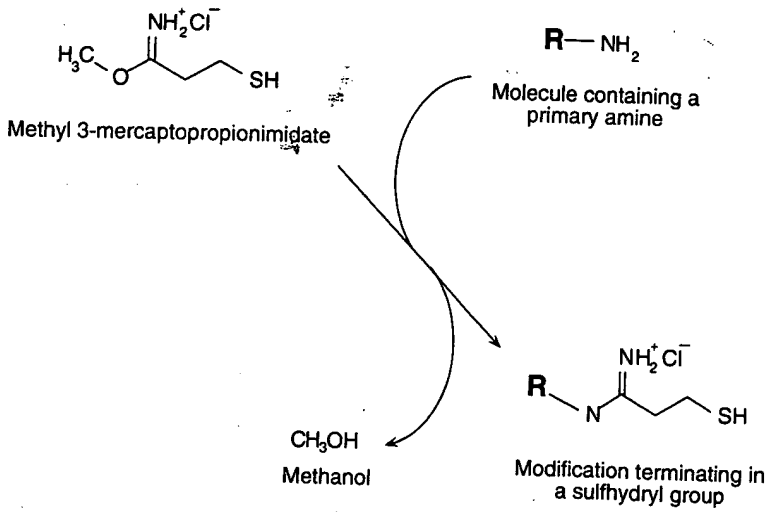


Figure 52 Thiolation of an amine-containing compound with methyl 3-mercaptopropionimidate. The modification preserves the positive charge on the primary amine.

this wavelength decreases. With addition of the dipeptide glycylglycine, the starting absorbance of a solution of Traut's reagent decreased over 80% within 20 min (Jue *et al.*, 1978). Thus, protein modification with 2-iminothiolane is very efficient and proceeds rapidly at slightly basic pH.

At high pH (10), Traut's reagent also is reactive with aliphatic and aromatic hydroxyl groups, although the rate of reaction with these groups is only about 0.01 that of primary amines. In the absence of amines, however, carbohydrates such as agarose or cellulose membranes can be modified to contain sulfhydryl residues (Alagon and

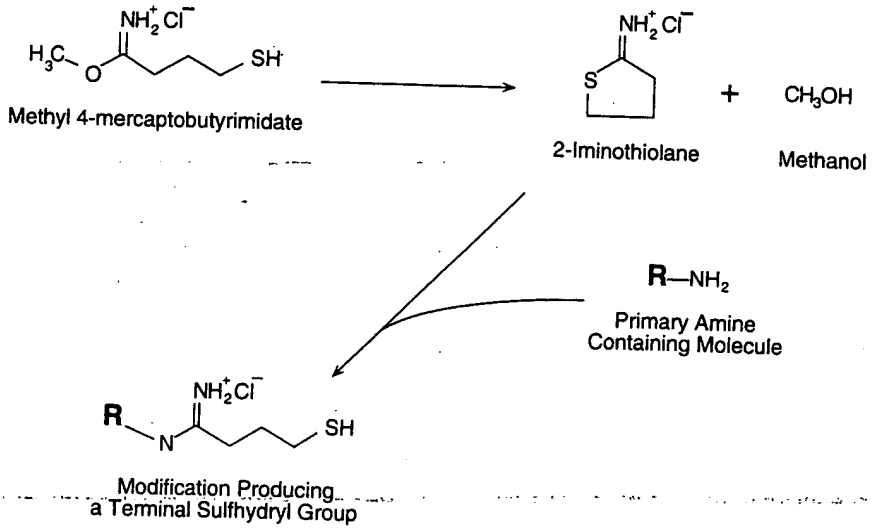


Figure 53 Methyl 4-mercaptobutyrimidate forms 2-iminothiolane, which can react with a primary amine to create a sulfhydryl group. The modification preserves the positive charge of the original amine.

King, 1980). Polysaccharides modified in this manner are effective in covalently cross-linking antibodies for use in immunoassay procedures.

Proteins modified with 2-iminothiolane are subject to disulfide formation on sulfhydryl oxidation. This can cause unwanted conjugation, potentially precipitating the protein. The addition of a metal chelating agent such as EDTA (0.01–0.1 *M*) will prevent metal-catalyzed oxidation and maintain sulfhydryl stability. In the presence of some serum proteins (i.e., BSA) a 0.1 *M* concentration of EDTA may be necessary to prevent metal-catalyzed oxidation, presumably due to the high contamination of iron from hemolyzed blood.

Traut's reagent has been used successfully in the investigation of ribosomal proteins (Sun *et al.*, 1974; Jue *et al.*, 1978; Kenny *et al.*, 1979; Blattler *et al.*, 1985 a, b; Lambert *et al.*, 1983), RNA polymerase (Hillel and Wu, 1977), and progesterone receptor subunits (Birnbaumer *et al.*, 1979), and in the synthesis of enzyme-labeled DNA hybridization probes (Ghosh, *et al.*, 1990). It is an excellent thiolation reagent for use in the preparation of immunotoxins (Chapter 11). Recently, it has been used to modify and introduce sulfhydryls into oligosaccharides from asparagine-linked glycans (Tarentino *et al.*, 1993).

Protocol

1. Prepare the protein or macromolecule to be thiolated in a non-amine-containing buffer at pH 8.0. For the modification of ribosomal proteins (often cited in the literature) use 50 m*M* triethanolamine hydrochloride, 1 m*M* $MgCl_2$, 50 m*M* KCl, pH 8. The magnesium and potassium salts are for stabilization of some ribosomal proteins. If other proteins are to be thiolated, the same buffer may be used without added salts for stabilization. Alternatively, 50 m*M* sodium phosphate, 0.15 *M* NaCl, pH 8, or 0.1 *M* sodium borate, pH 8.0 may be used. For the modification of polysaccharides, use 20 m*M* sodium borax, pH 10, to produce reactivity toward carbohydrate hydroxyl residues. Dissolve the protein to be modified at a concentration of 10 mg/ml in the reaction buffer of choice. Lower concentrations also may be used with a proportional scaling back of added 2-iminothiolane.
2. Dissolve the Traut's reagent (Pierce) in water at a concentration of 2 mg/ml (makes a 14.5 m*M* stock solution). The solution should be used immediately. For the modification of IgG at a concentration of 10 mg/ml using a 10-fold molar excess of Traut's reagent, add 45.8 µl of the stock solution to each milliliter of the protein solution.
3. React for 1 h at room temperature (a 4°C reaction temperature may be used successfully as well).
4. Purify the thiolated protein from unreacted Traut's reagent by dialysis or gel filtration using your buffer of choice (i.e., 20 m*M* sodium phosphate, 0.15 *M* NaCl, 1 m*M* EDTA, pH 7.2). The addition of EDTA to this buffer helps to prevent oxidation of the sulfhydryl groups and the resultant disulfide formation.
5. The degree of —SH modification may be determined using the Ellman's assay (Section 4.1).

When 2-iminothiolane is used to modify proteins in tandem with 4,4′-dipyridyl disulfide, a protected sulfhydryl can be introduced in a single step (King *et al.*, 1978). The simultaneous reaction between a protein, 2-iminothiolane, and 4,4′-dipridyl di-

sulfide yields a modification containing pyridyl disulfide groups. The pyridyl disulfide may be subsequently reduced with DTT to yield a free sulfhydryl. Pyridyl disulfides also are highly reactive toward sulfhydryls through disulfide interchange (Section 5.2). The protocol is a modification of the method of King *et al.* (1978).

Protocol

1. Dissolve 1–10 mg of a protein to be modified in 1.0 ml of 0.025 *M* sodium borate, pH 9.
2. Dissolve 2-iminothiolane in 0.025 *M* sodium borate to a concentration of 0.02 *M*.
3. Dissolve 4,4′-dipyridyl disulfide at a concentration of 2 mg/ml in acetonitrile.
4. Add 0.2 ml of (3) and 1.0 ml of (2) to the protein solution.
5. React for 2 h at room temperature.
6. Purify the modified protein by gel filtration or dialysis.

Occasionally, a protein modified in this manner will begin to precipitate as the reaction proceeds. Stopping the reaction earlier or adding a smaller quantity of modifying reagents may limit this effect.

Modification of Amines with SATA

A versatile reagent for introducing sulfhydryl groups into proteins is SATA, *N*-succinimidyl *S*-acetylthioacetate (Duncan *et al.*, 1983). The active NHS ester end of SATA reacts with amino groups in proteins and other molecules to form a stable amide linkage (Fig. 54) (Chapter 2, Section 1.4). The modified protein then contains a

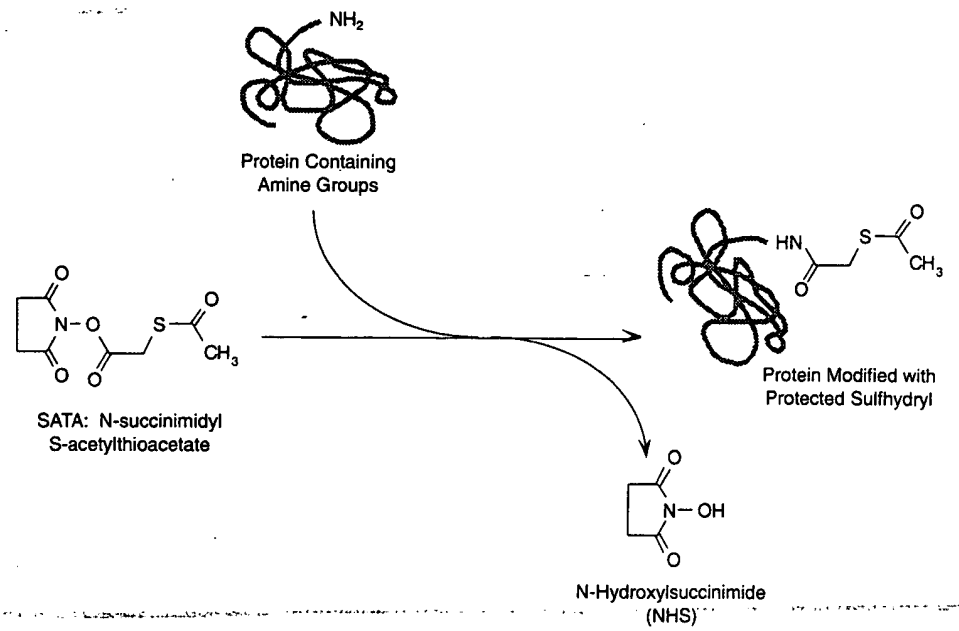
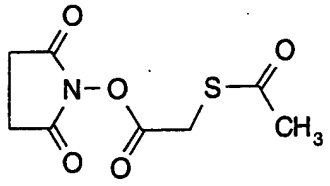


Figure 54 SATA can react with available amine groups in proteins and other molecules via its NHS ester end to form protected sulfhydryl derivatives.

protected sulfhydryl that can be stored without degradation and subsequently deprotected as needed with an excess of hydroxylamine (Fig. 55). Since the protecting group can be removed without adding disulfide reducing agents like DTT, disulfides indigenous to the native protein will not be affected. This is an important consideration if disulfides are vital to activity, such as in the case of some protein toxins.



SATA;
N-succinimidyl
S-acetylthioacetate
MW 231.2

SATA is often used to form antibody–enzyme conjugates utilizing maleimide-containing heterobifunctional cross-linking agents. Most polyclonal antibody molecules may be modified to contain up to about six SATA molecules per immunoglobulin with minimal effect on antigen binding activity. Some sensitive monoclonal antibodies, however, may be susceptible to modification and should be tested on a case-by-case basis. The modified antibody then may be deprotected and reacted with a maleimide-activated enzyme to form a conjugate useful in immunoassays (Chapter 10, Section 1.1). Conjugates formed using SATA are usually of low molecular weight with very few high-molecular-weight oligomers. They also maintain a bivalent antibody, ensuring a conjugate containing two antigen binding sites. This is an advantage over reduction schemes that break the antibody molecule into two heavy–light chain pairs to create sulfhydryls, since disulfide cleavage yields antibody fragments with only one antigen binding site.

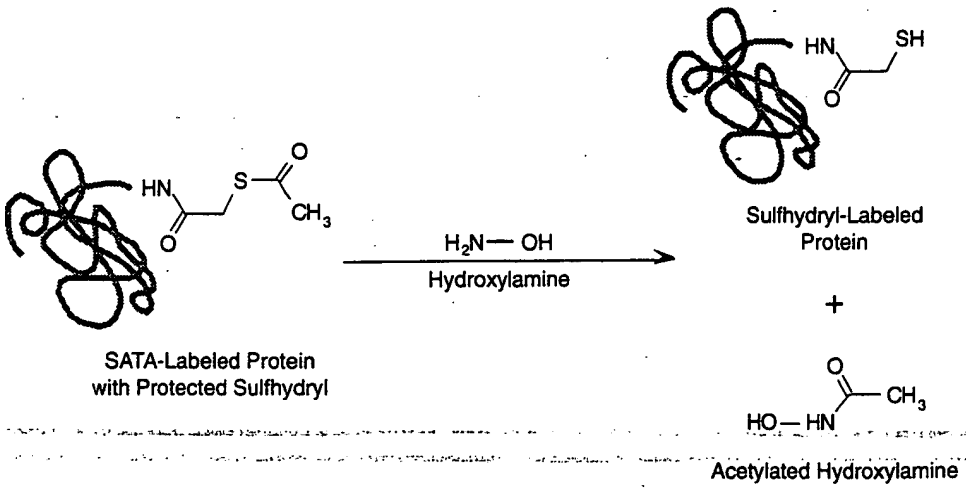


Figure 55 Deprotection with hydroxylamine of the acetylated thiol of SATA-modified proteins yields a free sulfhydryl group.

SATA has been used to form conjugates with avidin or steptavidin with excellent retention of activity (Chapter 13, Section 3.1). It has been used in the formation of a therapeutically useful toxin conjugate with recombinant CD4 (Ghetie *et al.*, 1990A).

SATA is freely soluble in many organic solvents. In use, it is typically dissolved as a stock solution in DMSO, DMF, or methylene chloride, and then an aliquot of this solution is added to an aqueous reaction mixture containing the protein to be modified.

The thiolation method described below is generally applicable for the modification of proteins with SATA, particularly for subsequent conjugation with a maleimide-activated secondary protein. The degree of modification described usually yields 3–4 mol of —SH groups per mole protein when thiolating immunoglobulins. Other macromolecules containing primary amines may be modified using a similar procedure. The degree of modification observed with other molecules may vary depending on the number of available primary amines and their relative reactivity. For comparison purposes, the molar ratio of SATA to immunoglobulin added to a reaction for the modification of rabbit polyclonal IgG versus the degree of sulfhydryl incorporation is illustrated in Fig. 56 (Sykaluk,, 1994).

The following protocol represents a generalized method for protein thiolation using SATA. For comparison purposes, contrast the variation of this SATA modification method as outlined in Chapter 10, Section 1.1 for use in the preparation of antibody–enzyme conjugates.

Protocol

1. Dissolve the protein to be thiolated at a concentration of 1–5 mg/ml in 50 m*M* sodium phosphate, pH 7.5, containing 1–10 m*M* EDTA. Other non-amine-containing buffers such as borate, Hepes, and bicarbonate also may be used as the reaction medium. The effective pH for the NHS ester modification reaction is in the range 7 to 9.
2. Dissolve the SATA reagent (Pierce) in DMSO at a concentration of 65 m*M* (15 mg/ml). Note: DMSO should be handled in a fume hood.

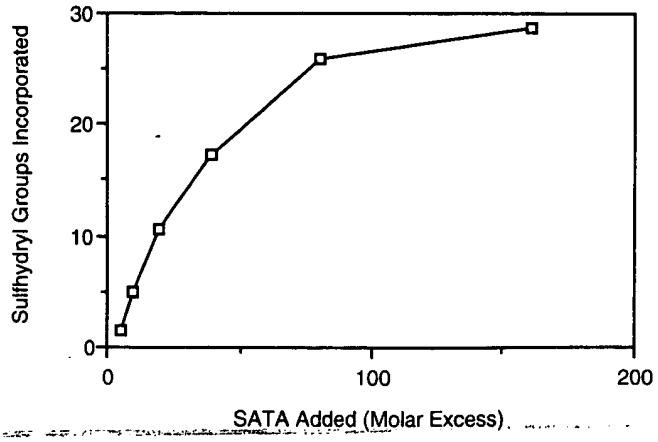


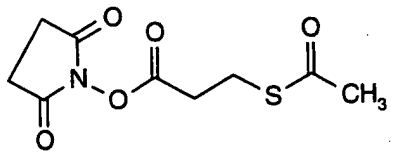
Figure 56 SATA modification of rabbit polyclonal IgG with the resultant sulfhydryl incorporation level.

3. Add 10 µl of the SATA solution to each milliliter of protein solution.
4. Mix and react for 30 min at room temperature.
5. Separate modified protein from unreacted SATA and reaction by-products by dialysis against 50 m*M* sodium phosphate, pH 7.5, containing 1 m*M* EDTA or by gel filtration on a Sephadex G-25 column (Pharmacia) using the same buffer.
6. Deprotect the acetylated —SH groups as needed by adding 100 µl of 0.5 *M* hydroxylamine hydrochloride in 50 m*M* sodium phosphate, 25 m*M* EDTA, pH 7.5, to each milliliter of the SATA-modified protein solution.
7. Mix and react for 2 h at room temperature.
8. Purify the sulfhydryl-modified protein by dialysis against 50 m*M* sodium phosphate, 1 m*M* EDTA, pH 7.5, or by gel filtration on a Sephadex G-25 column using the same buffer.

The deacetylated protein should be used immediately to prevent loss of sulfhydryl content through disulfide formation. The degree of —SH modification may be determined by performing an Ellman's assay (Section 4.1).

Modification of Amines with SATP

SATP, succinimidyl acetylthiopropionate, is an analog of SATA (Section 4.1) containing one additional carbon atom in length (Fuji *et al.*, 1985). The compound retains all the advantages of a protected sulfhydryl, including stability of the modified protein and selective release of the protecting group with hydroxylamine to free the sulfhydryl as needed (Fig. 57). SATP is soluble in DMF and methylene chloride. It is usually first solubilized in organic solvent and an aliquot added to an aqueous solution containing the macromolecule to be modified. It is particularly useful in adding an N-terminal —SH group at the completion of peptide synthesis.



SATP
Succinimidyl acetyl-
thiopropionate
MW 245

Protocol

1. Dissolve the protein or peptide to be thiolated at a concentration of 10 mg/ml in 50 m*M* sodium phosphate, pH 7.5, containing 1 m*M* EDTA. Other non-amine-containing buffers such as borate, Hepes, and bicarbonate also may be used as the reaction medium. The effective pH for the NHS ester modification reaction is in the range 7 to 9.
2. Dissolve the SATP reagent (Molecular Probes) in DMF at a concentration of 65 m*M* (16 mg/ml). Note: DMF should be handled in a fume hood.
3. Add 10 µl of the SATP solution to each milliliter of protein or peptide solution.
4. Mix and react for 8 h (or overnight) at room temperature.

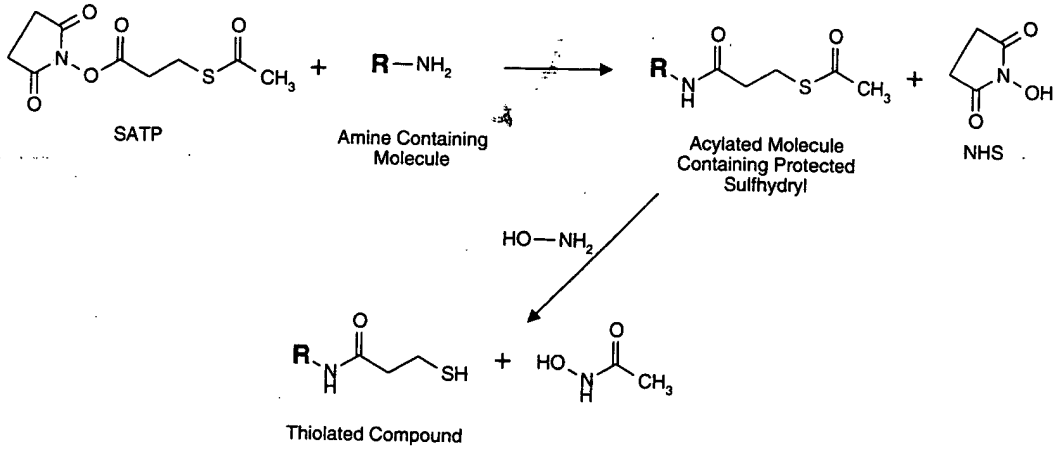


Figure 57 SATP reacts with amine-containing proteins or other molecules via its NHS ester end to create protected sulfhydryl derivatives in a manner similar to that of SATA. Deprotection can be done with hydroxylamine to free the thiol.

5. Separate modified protein from unreacted SATP and reaction by-products by dialysis against 50 m*M* sodium phosphate, pH 7.5, containing 1 m*M* EDTA or by gel filtration on a Sephadex G-25 column (Pharmacia) using the same buffer. If a peptide of low molecular weight is being modified, careful gel filtration using a matrix having a low exclusion limit will separate the peptide from the reaction by-products. In this case, use either Sephadex G-25 or Sephadex G-10 for the chromatography.
6. Deprotect the acetylated —SH groups as needed by adding 100 µl of 0.5 *M* hydroxylamine hydrochloride in 50 m*M* sodium phosphate, 25 m*M* EDTA, pH 7.5, to each milliliter of the SATP-modified protein solution.
7. Mix and react for 2 h at room temperature.
8. Purify the sulfhydryl-modified protein by dialysis against 50 m*M* sodium phosphate, 1 m*M* EDTA, pH 7.5, or by gel filtration on a Sephadex G-25 column using the same buffer. Again, if a peptide of low molecular weight is being modified, use gel filtration for purification.

The deacetylated protein should be used immediately to prevent loss of sulfhydryl content through disulfide formation. The degree of —SH modification may be determined by performing an Ellman's assay (Section 4.1).

Modification of Amines with SPDP

SPDP, *N*-succinimidyl 3-(2-pyridyldithio)propionate, is one of the most popular heterobifunctional cross-linking agents (Chapter 5, Section 1.1). The NHS ester end of SPDP reacts with amine groups to form an amide linkage, while the 2-pyridyldithiol group at the other end can react with sulfhydryl residues to form a disulfide linkage (Carlsson *et al.*, 1978). The cross-linker is used extensively to form immunotoxin conjugates for *in vivo* administration (Chapter 11, Section 2.1). The reagent is also useful in creating sulfhydryls in proteins and other molecules. Once modified with

SPDP, a protein can be treated with DTT (or other disulfide reducing agents, see Section 4.1) to release the pyridine-2 thione leaving group and form the free sulfhydryl (Fig. 58). The terminal —SH group then can be used to conjugate with any cross-linking agents containing sulfhydryl-reactive groups, such as maleimide or iodoacetyl (for covalent conjugation) or 2-pyridyldithiol groups (for reversible conjugation).

There are three forms of SPDP analogs currently commercially available (Pierce Chemical): the standard SPDP, a long-chain version designated LC-SPDP, and a water-soluble, sulfo-NHS form also containing a extended chain, called Sulfo-LC-SPDP (Chapter 5, Section 1.1). The main disadvantage to using SPDP to create sulfhydryls is the necessity of using a reducing agent to remove the pyridine-2-thione group. Reducing agents will also affect indigenous disulfides within a protein molecule, cleaving and reducing them. This method therefore works well for proteins containing no sulfhydryls or no disulfides that are critical to function, but it may cause loss of activity or subunit breakdown in proteins containing essential disulfides.

The following procedure is similar to the method of Cumber *et al.* (1985), but with some modifications.

Protocol

1. Dissolve the protein or macromolecule to be thiolated at a concentration of 10 mg/ml in 50 m*M* sodium phosphate, 0.15 *M* NaCl, pH 7.2. Other non-amine-containing buffers such as borate, Hepes, and bicarbonate also may be used in this reaction. The effective pH for the NHS ester modification reaction is in the range 7.0 to 9.0.

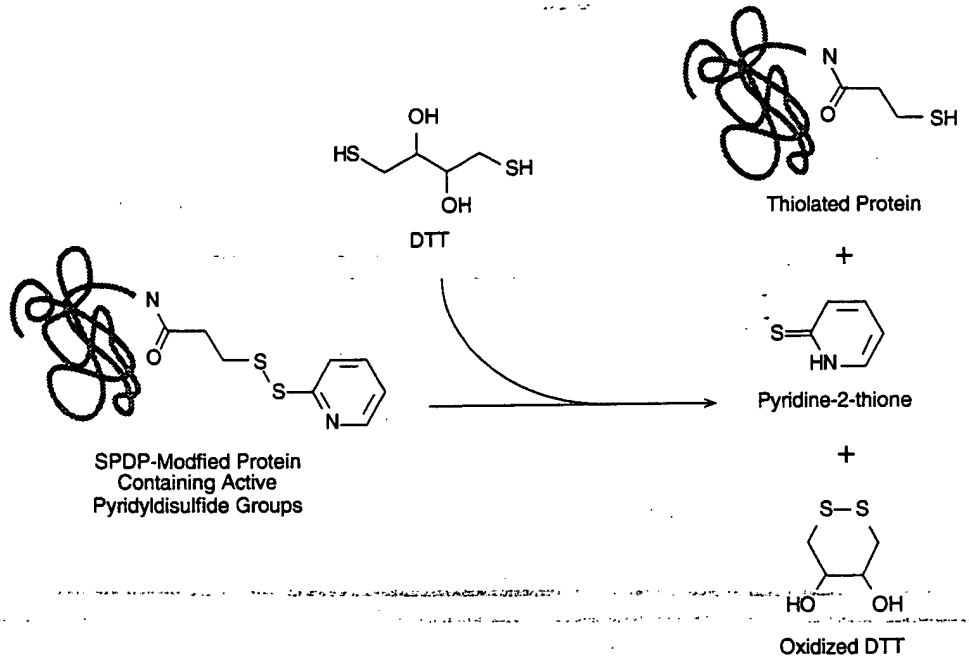


Figure 58 SPDP-modified proteins can be reduced with DTT to yield free sulfhydryl groups for conjugation.

2. Dissolve SPDP (Pierce) at a concentration of 6.2 mg/ml in DMSO (makes a 20 m*M* stock solution). Alternatively, LC-SPDP may be used and dissolved at a concentration of 8.5 mg/ml in DMSO (also makes a 20 m*M* solution). If the water-soluble Sulfo-LC-SPDP is used, a stock solution in water may be prepared just prior to adding an aliquot to the thiolation reaction. In this case, prepare a 10 m*M* solution of Sulfo-LC-SPDP by dissolving 5.2 mg/ml in water. Since an aqueous solution of the cross-linker will degrade by hydrolysis of the sulfo-NHS ester, it should be used quickly to prevent significant loss of activity. If a sufficiently large amount of protein will be modified to allow accurate weighing of Sulfo-LC-SPDP, the solid may be added directly to the reaction mixture without preparing a stock solution in water.
3. Add 25 µl of the stock solution of either SPDP or LC-SPDP in DMSO to each milliliter of the protein to be modified. If Sulfo-LC-SPDP is used, add 50 µl of the stock solution in water to each milliliter of protein solution.
4. Mix and react for at least 30 min at room temperature. Longer reaction times, even overnight, will not adversely affect the modification.
5. Purify the modified protein from reaction by-products by dialysis or gel filtration using 50 m*M* sodium phosphate, 0.15 *M* NaCl, pH 7.2.
6. To release the pyridine-2-thione leaving group and form the free sulfhydryl, add DTT (Pierce) at a concentration of 0.5 mg DTT per milligram of modified protein. A stock solution of DTT may be prepared to make it easier to add it to a small amount of protein solution. In this case, dissolve 20 mg of DTT per milliliter of 0.1 *M* sodium acetate, 0.1 *M* NaCl, pH 4.5. Add 25 µl of this solution per milligram of modified protein. Release of pyridine-2-thione can be followed by its characteristic absorbance at 343 nm ($\varepsilon = 8.08 \times 10^3\ M^{-1}cm^{-1}$).
7. Mix and react at room temperature for 30 min.
8. Purify the thiolated protein from excess DTT by dialysis or gel filtration using 50 m*M* sodium phosphate, 0.15 *M* NaCl, 1 m*M* EDTA, pH 7.2. The modified protein should be used immediately in a conjugation reaction to prevent sulfhydryl oxidation and formation of disulfide cross-links.

Modification of Amines with SMPT

SMPT, succinimidyloxycarbonyl-α-methyl-α-(2-pyridyldithio)toluene, contains an NHS ester end and a pyridyldisulfide end similar to those of SPDP, but its hindered disulfide makes conjugates formed with this reagent more stable (Thorpe *et al.*, 1987) (Chapter 5, Section 1.2). The reagent is especially useful in forming immunotoxin conjugates for *in vivo* administration (Chapter 11, Section 2.1). A water-soluble analog of this cross-linker containing an extended spacer arm is also commercially available as Sulfo-LC-SMPT (Pierce).

SMPT or Sulfo-LC-SMPT may be used as thiolation reagents by first reacting its NHS ester end with an amine-containing molecule and then releasing the pyridine-2-thione leaving group with DTT to free the sulfhydryl (Fig. 59). The disadvantage of this approach is the necessity of using a reducing agent to create the —SH group modification. This method of thiolation only should be used if there are no disulfides in the target molecule that are critical to function. If a reductant cannot be used, choose a thiolation method that does not need DTT treatment, such as the use of Traut's reagent or SATA (Section 4.1).

Since SMPT is not soluble in aqueous solutions it must be first dissolved in organic

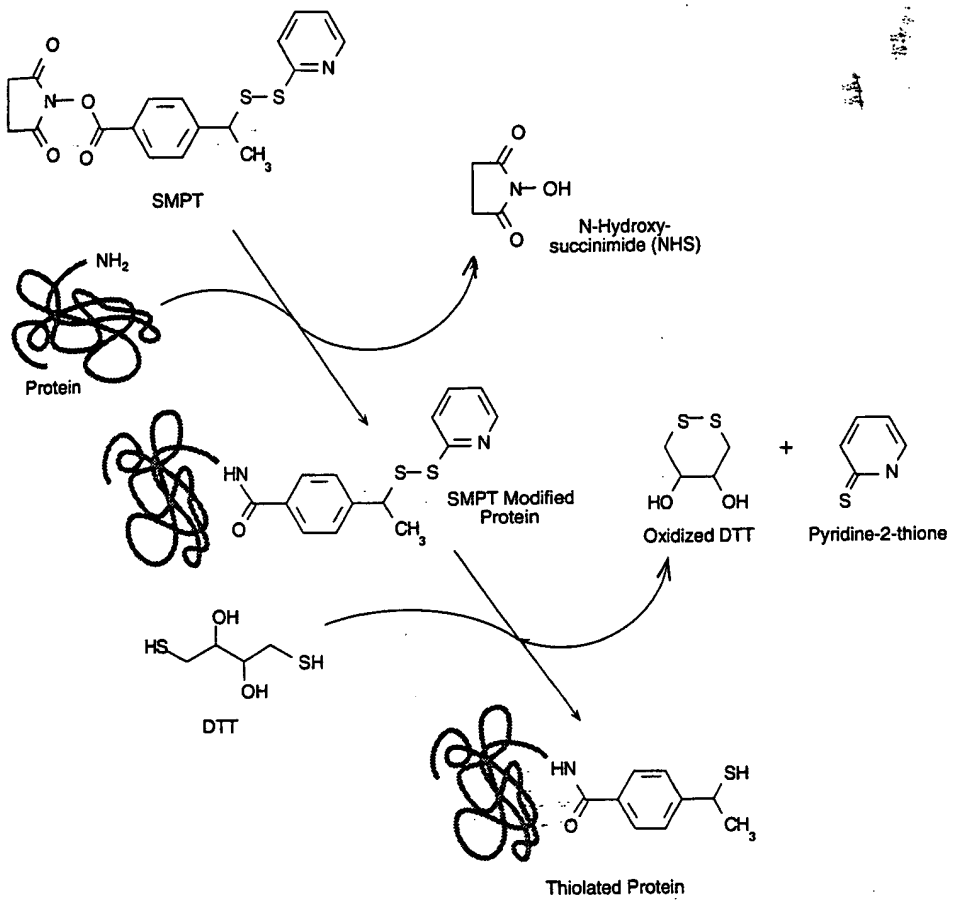


Figure 59 SMPT can be used to modify the amine groups of proteins to form disulfide intermediates. The disulfides can be reduced with DTT to create free thiols for subsequent conjugation purposes.

solvent and an aliquote of this stock solution transferred to the reaction solution. The reagent is soluble in DMF and DMSO, but is much more stable in solutions of acetonitrile. A stock solution of SMPT in acetonitrile may be kept frozen without loss of activity. The NHS ester of SMPT also is extraordinarily stable to hydrolysis in water. Even when an SMPT/acetonitrile aliquot is added to an aqueous solution and stored at room temperature, SMPT will only lose about 5% of its activity after 16 h. By contrast, other NHS esters usually have half-lives of only 2–6 h in aqueous environments.

Sulfo-LC-SMPT is not as stable as SMPT. The sulfo-NHS ester is more susceptible to hydrolysis in aqueous solutions and the pyridyldisulfide group is more easily reduced to the free sulfhydryl. Stock solutions of Sulfo-LC-SMPT may be prepared in water, but should be used immediately to prevent loss of amine coupling ability.

Protocol

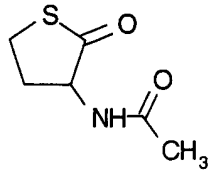
1. Dissolve the protein or macromolecule to be thiolated at a concentration of 10 mg/ml in 50 m*M* sodium phosphate, 0.15 *M* NaCl, pH 7.2. Other non-amine-containing buffers such as borate, Hepes, and bicarbonate also may be used as

the reaction medium. The effective pH for the NHS ester modification reaction is in the range of 7 to 9.

2. Dissolve SMPT (Pierce) at a concentration of 7.7 mg/ml in acetonitrile (makes a 20 m*M* stock solution). Alternatively, the water-soluble Sulfo-LC-SMPT may be used and dissolved at a concentration of 6 mg/ml in water (makes a 10 m*M* solution). This should be done just prior to adding an aliquot to the thiolation reaction. Since an aqueous solution of the cross-linker will degrade by hydrolysis of the sulfo-NHS ester, it should be used quickly to prevent significant loss of activity. If a sufficiently large amount of protein will be modified to allow accurate weighing of Sulfo-LC-SMPT, the solid may be added directly to the reaction mixture without preparing a stock solution in water.
3. Add 25 µl of the stock solution of SMPT in acetonitrile to each milliliter of the protein to be modified. If Sulfo-LC-SMPT is used, add 50 µl of the stock solution in water to each milliliter of protein solution.
4. Mix and react for at least 30 min at room temperature. Longer reaction times, even overnight, will not adversely affect the modification.
5. Purify the modified protein from reaction by-products by dialysis or gel filtration using 50 m*M* sodium phosphate, 0.15 *M* NaCl, pH 7.2.
6. To release the pyridine-2-thione leaving group and form the free sulfhydryl, add DTT (Pierce) at a concentration of 0.5 mg DTT per milligram of modified protein. A stock solution of DTT may be prepared to make it easier to add it to a small amount of protein solution. In this case, dissolve 20 mg of DTT per milliliter of 0.1 *M* sodium acetate, 0.1 *M* NaCl, pH 4.5. Add 25 µl of this solution per milligram of modified protein. Release of pyridine-2-thion can be followed by its characteristic absorbance at 343 nm ($\varepsilon = 8.08 \times 10^3\ M^{-1}cm^{-1}$).
7. Mix and react at room temperature for 30 min.
8. Purify the thiolated protein from excess DTT by dialysis or gel filtration using 50 m*M* sodium phosphate, 0.15 *M* NaCl, 1 m*M* EDTA, pH 7.2. The modified protein should be used immediately in a conjugation reaction to prevent sulfhydryl oxidation and formation of disulfide cross-links.

Modification of Amines with N-Acetylhomocysteinethiolactone

N-Acetylhomocysteinethiolactone (also called citiolone or 2-acetamido-4-mercaptobutyric acid) is a cyclic derivative of homocysteine containing a blocked α-amino group. The compound can react with primary amines in a ring-opening reaction to create free sulfhydryl modifications (Fig. 60). It was originally used as a reagent for insolubilizing antibodies. Later, it was immobilized on an amine-containing matrix to form a disulfide reducing support for cleaving cystine residues in peptides and proteins



N-Acetyl Homocysteine
Thiolactione
MW 159

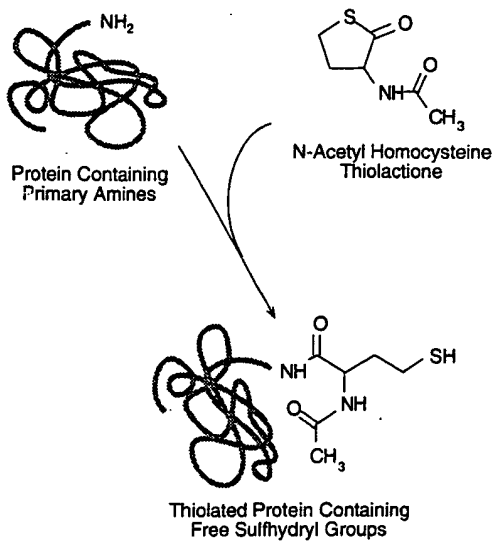


Figure 60 *N*-Acetylhomocysteinethiolactone spontaneously reacts with amine groups on proteins to create sulfhydryl groups.

(Eldjarn and Jellum, 1963; Jellum, 1964) (see Section 4.1). The thiolation reaction of amine-containing macromolecules proceeds much like the reaction for 2-iminothiolane. Nucleophilic attack occurs at the carbonyl, cleaving the thiolactone and producing an amide linkage with the target molecule, while at the same time creating the free sulfhydryl (Benesch and Benesch, 1956, 1958). *N*-Acetylhomocysteine is soluble in aqueous buffers.

Thiolation of peptides and other small molecules containing amines proceeds easily with *N*-acetylhomocysteinethiolactone. However, protein modification often results in much lower yields unless the reaction is done for extended periods at pH 10–11.

It has been found that silver ions catalyze the thiolation process with proteins, allowing the reaction to be completed rapidly at physiological pH (Benesch and Benesch, 1958). The addition of an equal molar concentration of $AgNO_3$ forms an insoluble complex with the thiolactone, and this in turn reacts with protein amines.

Protocol

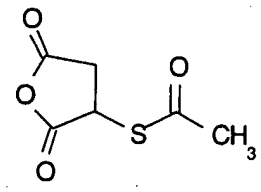
1. Dissolve the amine-containing molecule to be thiolated at a concentration of 10 mg/ml in cold (4°C) 1 *M* sodium bicarbonate (reaction buffer). For proteins, dissolve them in deionized water at a pH of 7.0–7.5, at room temperature. Note: the presence of some buffer salts, like phosphate or carbonate, are incompatible with silver nitrate.
2. Add *N*-acetylhomocysteinethiolactone (Aldrich) to the bicarbonate reaction mixture to obtain a concentration representing a 10- to 20-fold excess over the amount of amines present. For protein thiolation, add the same molar excess of thiolactone reagent to the water reaction medium; then slowly add an equivalent molar quantity of silver nitrate ($AgNO_3$). Maintain the pH at 7.0–7.5 with periodic addition of NaOH.

3. For the bicarbonate reaction, gently mix for 20 h at 4°C. For the silver-catalyzed reaction, continue for 1 h or until the silver complex has fully dissolved.
4. To remove the silver mercaptide formed from the facilitated protein thiolation reaction, add an excess of thiourea to convert all the silver into a soluble Ag(thiourea)$_2^+$ complex and free the sulfhydryl modifications.
5. Remove unreacted *N*-acetylhomocysteinethiolactone and reaction by-products by gel filtration or dialysis against 10 m*M* sodium phosphate, 0.15 *M* NaCl, 10 m*M* EDTA, pH 7.2. Other buffers suitable for individual protein stability may be used as desired. For the silver nitrate-containing reaction, removal of the silver–thiourea complex may be done by adsorption onto Dowex 50, and the protein subsequently eluted from the resin by 1 *M* thiourea. Removal of the thiourea then may be done by gel filtration or dialysis.

Including EDTA in the final preparation inhibits metal-catalyzed oxidation of the sulfhydryl groups to disulfides. The modified peptide or protein should be used immediately to ensure full sulfhydryl reactivity.

Modification of Amines with SAMSA

S-Acetylmercaptosuccinic anhydride, or SAMSA, is an amine-reactive reagent containing a protected sulfhydryl much like SATA described previously. The anhydride portion opens in response to the attack of an amine nucleophile, yielding an amide linkage (Weston *et al.*, 1980; Klotz and Heiney, 1962; Klots and Keresztes-Nagy, 1962). The ring-opening reaction, however, does produce a free carboxylate group that lends a negative charge to the modified molecule where once there was a positive charge (Fig. 61). This charge reversal may affect the conformation and activity of some sensitive proteins. After the initial modification step, the thiolated derivative is formed by releasing the acetylated sulfhydryl protecting group with hydroxylamine.



SAMSA;
S-Acetylmercaptosuccinic
Anhydride
MW 174

Protocol

1. Dissolve the protein or other amine-containing macromolecule in 0.1 *M* sodium phosphate, 0.15 *M* NaCl, pH 7.5, at a concentration of 5 mg/ml.
2. Dissolve SAMSA in DMF at a concentration of 25 mg/ml.
3. Add 20 µl of the stock SAMSA solution to each milliliter of the protein solution, with mixing.
4. React at room temperature for 30 min.
5. Remove excess reagent and reaction by-products by dialysis or gel filtration

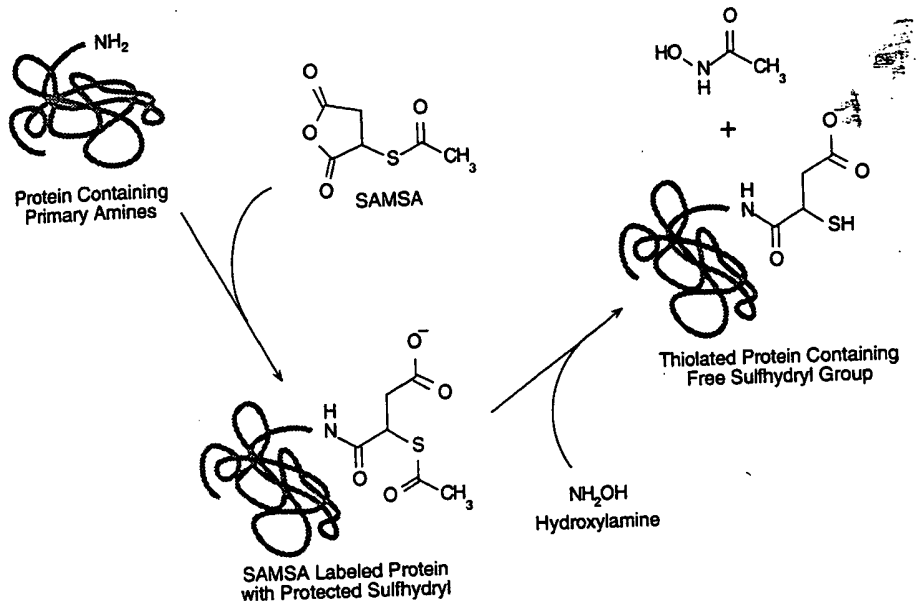


Figure 61 SAMSA is an anhydride compound containing a protected thiol. Reaction with protein amine groups yields amide bond linkages. Deprotection of the acetylated thiol produces free sulfhydryl groups for conjugation.

using 0.1 *M* sodium phosphate, 0.15 *M* NaCl, 10 m*M* EDTA, pH 7.5. For chromatographic separation use Sephadex G-25 (Pharmacia) or the equivalent. The SAMSA-modified protein may be stored at -20°C until needed.

6. To deprotect the acetylated sulfhydryl group of SAMSA-modified proteins, add 100 µl of 0.5 *M* hydroxylamine hydrochloride in 50 m*M* sodium phosphate, 25 m*M* EDTA, pH 7.5, to each milliliter protein solution.
7. Mix and react for 2 h at room temperature.
8. Purify the sulfhydryl-modified protein by dialysis against 50 m*M* sodium phosphate, 1 m*M* EDTA, pH 7.5, or by gel filtration on a Sephadex G-25 column using the same buffer.

The deacetylated protein should be used immediately to prevent loss of sulfhydryl content through disulfide formation. The degree of —SH modification may be determined by performing an Ellman's assay (Section 4.1).

Modification of Aldehydes or Ketones with AMBH

AMBH (2-acetamido-4-mercaptobutyric acid hydrazide) is a unique hydrazide derivative that can thiolate aldehydes and ketones to form reactive sulfhydryl groups (Taylor and Wu, 1980). It is particularly useful in converting oxidized carbohydrates. In this respect, glycoproteins or other carbohydrate- and diol-containing molecules may be treated with sodium periodate under mild conditions to form aldehyde residues (see Section 4.4). The aldehydes readily react with the hydrazide groups of AMBH to form hydrazone linkages, leaving a free terminal sulfhydryl residue to use in further conjugation reactions (Fig. 62).

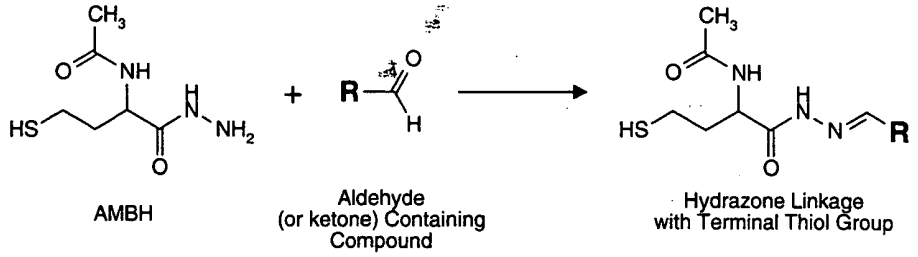
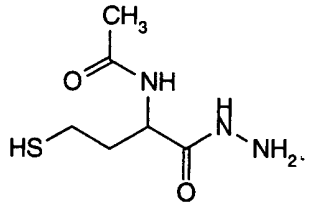


Figure 62 AMBH is a hydrazide-containing compound that reacts with carbonyl groups to form hydrazone bonds. The free thiol can be used for subsequent conjugation reactions.



AMBH
2-Acetamido-4-mercaptobutyric
acid hydrazide
MW 191

Protocol

1. Dissolve an aldehyde-containing macromolecule to be modified (i.e., a periodate-oxidized glycoprotein) in 0.01 *M* sodium phosphate, 0.15 *M* NaCl, pH 7.4, containing 1 m*M* EDTA. A suitable concentration range for a protein is 1–10 mg/ml.
2. Add a 10–fold molar excess of AMBH (predissolved in ethanol) (Molecular Probes) over the expected amounts of aldehydes to be modified.
3. React for 2 h at room temperature.
4. Purify the modified protein by gel filtration

Modification of Carboxylates or Phosphates with Cystamine

Cystamine is decarboxylated cystine [or 2,2′-dithiobis(ethylamine)], a small disulfide-containing molecule with primary amines at both ends. This versatile reagent can be used in several conjugation techniques. Cystamine may be used to introduce sulfhydryl residues in proteins, nucleic acids, and other molecules, or as the active species in disulfide exchange cross-linking reactions, or in reversible conjugation procedures. The reagent can be used to create sulfhydryl groups in proteins or other molecules by first conjugating its terminal amino groups with the carboxylates on a target molecule using the carbodiimide reaction (Chapter 2, Section 1.11 and Chapter 3, Section 1). Subsequent reduction of the disulfide group liberates the free sulfhydryl (Section 4.1) (Fig. 63). This same modification procedure also can be used to introduce sulfhydryl residues at the 5′ phosphate group of DNA (Chu *et al.*, 1986; Ghosh *et al.*, 1990). The

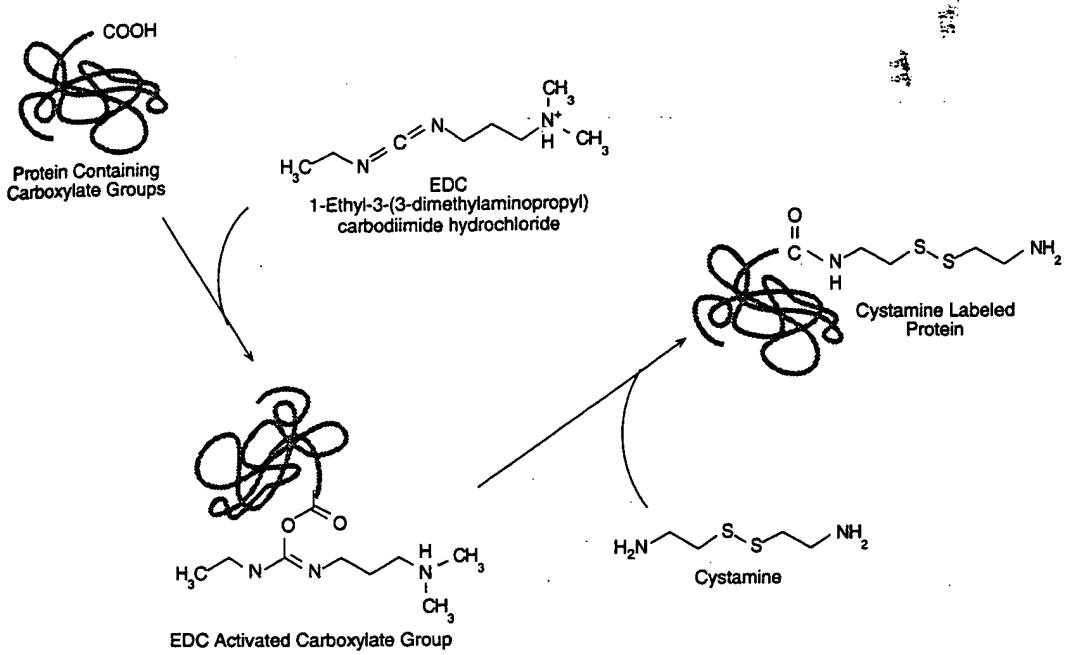
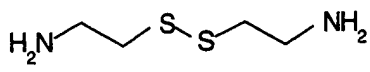


Figure 63 Cystamine may be used to label protein carboxylate groups using the water-soluble carbodiimide EDC.

carbodiimide activates the phosphate and the amines of cystamine may then react with this active species to form a phosphoramidate bond (Chapter 17, Section 2.2) (Fig. 64). Specific labeling of DNA probes only at the 5′ end is possible using this technique.



Cystamine;
2,2'-dithiobis(ethylamine)
MW 152

The carbodiimide of choice used to couple cystamine to carboxylate- or phosphate-containing molecules is most often the water-soluble carbodiimide EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; Chapter 3, Section 1.1). This reagent rapidly reacts with carboxylates or phosphates to form an active complex highly reactive toward primary amines. The reaction is efficient from pH 4.7 to 7.5, and a variety of buffers may be used, providing they do not contain competing groups.

Cystamine also is used as an activating reagent for disulfide exchange reactions. In this procedure, the reagent is used to modify one of two proteins to be conjugated. The cystamine-modified protein is then mixed with the other protein that contains, or is thiolated to contain, a sulfhydryl group. By disulfide exchange, the sulfhydryl-containing molecule cleaves the disulfide of the cystamine-modified protein, releasing 2-mercaptoethylamine and forming a disulfide cross-link (Fig. 65).

Using this approach, EGF has been successfully conjugated by disulfide exchange to

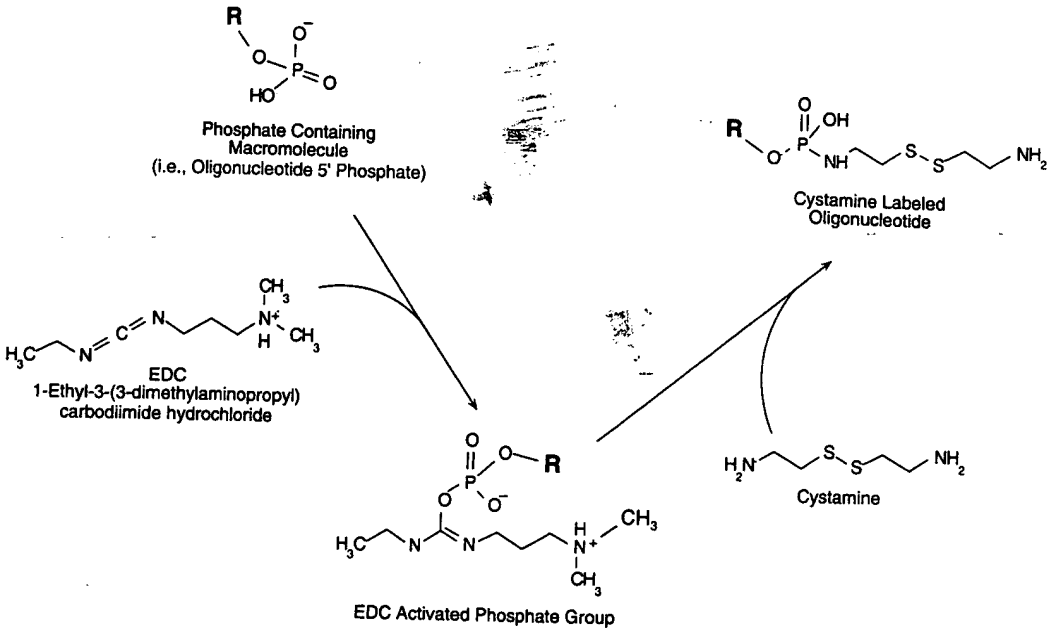


Figure 64 Cystamine may be used to label phosphate groups, such as on nucleic acids, via a carbodiimide reaction using EDC. The resultant phosphoramidate linkage is a common way to modify oligonucleotides at the 5′ end.

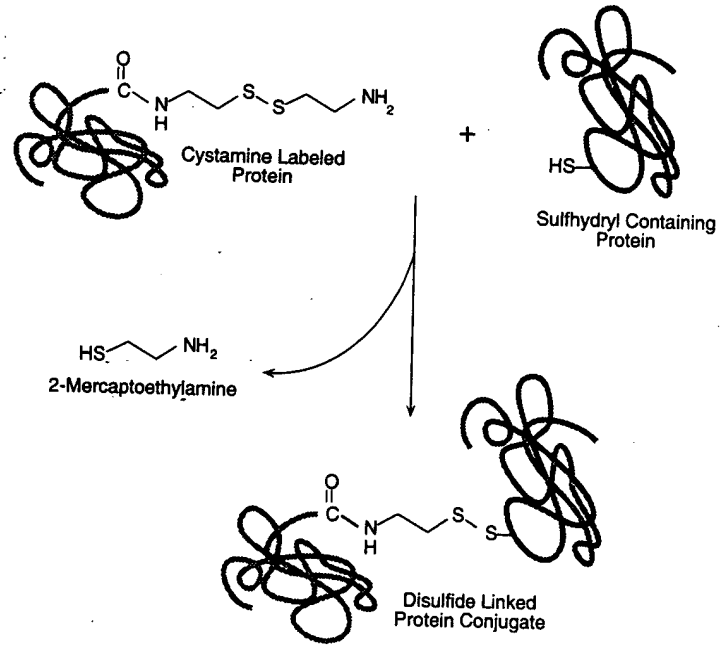


Figure 65 The disulfide group of a cystamine-modified protein may undergo disulfide interchange reactions with another sulfhydryl-containing protein to yield a disulfide-linked conjugate.

the A chain of diphtheria toxin (Shimisu *et al.*, 1980). A cystaminyl derivative of insulin also could be conjugated to the A chain of diphtheria toxin by this method (Miskimins and Shimizu, 1979). Other references to disulfide exchange using cystamine include Oeltmann and Forbes (1981) and Bacha *et al.* (1983), who prepared antibody–toxin and peptide–toxin conjugates, respectively.

Finally, cystamine may be used to conjugate two macromolecules through its terminal amine groups. In this case, the internal disulfide bridge remains intact, forming a reversible conjugate of the two molecules through reduction of the disulfide bond. Using this approach, the first molecule is modified with cystamine by use of the EDC reaction. A second molecule is then reacted with the free amines of cystamine on the first molecule by use of an amine-reactive chemistry. Typically, this reaction scheme is used if the first molecule initially contains no reactive amines and the second molecule is often an amine-reactive fluorescent tag or other probe. For instance, DNA probes may be cystamine-modified through their 5′ phosphate group using this method and amine-reactive biotin labels subsequently attached. The biotin label is then reversible by virtue of the cystamine cross-bridge through simple disulfide reduction.

Modification of Proteins with Cystamine

The following protocol is useful for the modification of proteins with cystamine with subsequent reduction to create the free sulfhydryl.

Protocol

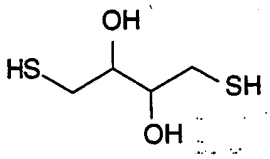
1. Dissolve the protein to be modified at a concentration of 10 mg/ml in a buffer having a pH between 4.7 and 7.5. Avoid buffers or other components containing groups competing with the carbodiimide reaction (i.e., carboxylates or amines). For the lower pH conditions, 0.1 *M* MES, pH 4.7, works best. For a physiological pH environment, 0.1 *M* sodium phosphate, 0.15 *M* NaCl, pH 7.2, also will give good incorporation of cystamine. For other concentrations of protein in solution, proportionally adjust the amount of reagents added.
2. Dissolve cystamine (Aldrich) in the reaction buffer at a concentration of 2.25 mg/ml (10 m*M*). Add an aliquot of this solution to the protein solution to be modified. Use about a 10- to 20-fold molar excess of cystamine over the amount of protein present. For a protein of MW 100,000 at a concentration of 10 mg/ml, add 10 μl of the stock cystamine solution to each milliliter of protein solution to obtain a 10-fold molar excess.
3. Add EDC (Pierce) to the solution prepared in (2) to obtain at least a five-fold molar excess over the amount of cystamine present. React for 2 h at room temperature.
4. Separate excess cystamine and EDC (and reaction by-products) from the modified protein by dialysis or gel filtration using 10 m*M* sodium phosphate, 0.15 *M* NaCl, pH 7.2. A desalting column may be used for the gel filtration procedure (i.e., Sephadex G-25 from Pharmacia).
5. To reduce the disulfide groups, add DTT (Pierce) at a concentration of 0.5 mg DTT per milligram of modified protein. A stock solution of DTT may be prepared to make it easier to add it to a small amount of protein solution. In this case, dissolve 20 mg of DTT per milliliter of 0.1 *M* sodium acetate, 0.1 *M* NaCl, pH 4.5. Add 25 μl of this solution per milligram of modified protein.

native protein structure, many buried disulfides would remain unaffected by the reductants.

The following reducing agents represent the most popular options for cleaving disulfide bonds. Their properties and use vary widely. The decision of which reagent is best often is governed by the molecule being reduced and the potential application. Careful review of these properties may sway the success or failure of a conjugation protocol.

Cleland's Reagent: DTT and DTE

Dithiothreitol (DTT) and dithioerythritol (DTE) are the *trans* and *cis* isomers of the compound 2,3-dihydroxy-1,4-dithiolbutane. The reducing potential of these versatile reagents was first described by Cleland in 1964. Due to their low redox potential (-0.33 V) they are able to reduce virtually all accessible biological disulfides and maintain free thiols in solution despite the presence of oxygen. The compounds are fully water-soluble with very little of the offensive odor of the 2-mercaptoethanol they were meant to replace. Since Cleland's original report, literally hundreds of references have appeared citing the use of mainly DTT for the reduction of cystine and other forms of disulfides.



DTT;
Dithiothreitol
MW 154.25

The unique characteristics of DTT and DTE are mainly reflected in their ability to form intramolecular ring structures upon oxidation. Disulfide reductants such as 2-mercaptoethanol, 2-mercaptoethylamine, glutathione, thioglycolate, and 2,3-dimercaptopropanol cleave disulfide bonds in a two-step reaction that involves the formation of a mixed disulfide (Fig. 66). In the second stage of the reducing process, the mixed disulfide is cleaved by another molecule of reductant, freeing the sulfhydryl and forming a dimer of the reducing agent through the formation of a intermolecular disulfide bond. For simple reductants containing only one thiol, the equilibrium for disulfide exchange is nearly equivalent for the reductant and target protein. Thus, monothiol compounds are usually required in extreme excess to drive the reaction to completion.

The presence of two sulfhydryl groups in DTT and DTE, however, allows the formation of a favored cyclic disulfide during the course of target protein reduction (Fig. 67). This drives the equilibrium toward the reduction of target disulfides. Therefore, complete reduction is possible with much lower concentrations of DTT or DTE than when using monothiol systems.

As with all reductants, DTT and DTE will reduce disulfides only if they are accessible. The three-dimensional structure of a protein molecule often contains disulfides buried deep in the inner structure of the polypeptide chains. A protein retaining its native conformation is frequently protected from complete reduction. In the absence

efficiently reduces all protein disulfides in the antibody and allows subunit separation for analysis (Konigsberg, 1972).

DTT also may be used to cleave disulfide-containing modification and cross-linking reagents. For thiolation procedures, DTT may be used to remove a dithiopyridyl group or cleave other disulfides to produce a free sulfhydryl. In this case, the presence of a denaturant usually is not required to access and reduce the disulfide of the modification reagent. Similarly, disulfides of cross-linking agents may be reduced after two macromolecules have been conjugated to release them as desired. This technique is often used to analyze receptor–ligand interactions or to discover how two proteins associate *in vivo*.

Complete Reduction of Disulfides in Protein Molecules Using DTT

Protocol

1. Dissolve a disulfide-containing protein or peptide at a concentration of 1–10 mg/ml in 6 *M* guanidine hydrochloride, 0.01 *M* sodium phosphate, 0.15 *M* NaCl, pH 7.4. Alternative denaturant conditions may be used [i.e., 8 *M* urea or 2.3% (w/w) SDS] along with any other buffer salts and pH values desired. A pH between 7.0 and 8.1 usually works best.
2. Add DTT (Pierce) to a final concentration of 10–100 m*M*.
3. Incubate for 2 h at room temperature. For some buried disulfides to become exposed and fully reduced, it may be necessary to heat the solution (in a capped test tube) at 50°C for 30 min. Some procedures use a 2-min incubation in a boiling water bath to completely denature the protein.
4. For removal of excess DTT, a protein of molecular weight greater than 5000 may be isolated by gel filtration using Sephadex G-25. To maintain the stability of the exposed sulfhydryl groups, include 1–10 m*M* EDTA in the chromatography buffer. The presence of oxidized DTT can be monitored during elution by measuring the absorbance at 280 nm. The protein should elute in the first peak and the DTT reaction products in the second peak.

Use of DTT to Cleave Disulfide-Containing Cross-linking Agents

The following method may be used to reduce the disulfide bonds of some cross-linking agents, thus cleaving conjugated proteins. This procedure will reduce the pyridyl disulfide group of SPDP (Section 4.1) to create a thiolated species. It also may be used to reduce partially the indigenous disulfides in some protein molecules. In this regard, DTT under nondenaturing conditions has been used to reduce selectively the disulfides between the heavy chains of immunoglobulin G (Edelman *et al.*, 1968). Without an added denaturant to open the polypeptide chain, internally buried disulfides typically will remain unreduced.

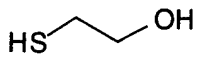
Protocol

1. Dissolve a cross-linked protein or peptide that has been conjugated with the use of a disulfide containing cross-linker at a concentration of 1–10 mg/ml in 0.01 *M* sodium phosphate, 0.15 *M* NaCl, pH 7.4. Alternative buffer conditions and pH levels may be used; however, a pH between 7.0 and 8.1 usually works best.

2. Add DTT to a final concentration of 1–10 m*M*.
3. Incubate for 2 h at room temperature.
4. For removal of excess DTT, a protein of molecular weight greater than 5000 may be isolated by gel filtration using Sephadex G-25. To maintain the stability of the exposed sulfhydryl groups, include 10 m*M* EDTA in the chromatography buffer. The presence of oxidized DTT can be monitored during elution by measuring the absorbance at 280 nm. The protein should elute in the first peak and the DTT reaction products in the second peak.

2-Mercaptoethanol

2-Mercaptoethanol is one of the most common agents used for disulfide reduction. Sometimes referred to as β-mercaptoethanol, it is a clear, colorless liquid with an extremely strong odor. All operations with this chemical should be performed in a well-ventilated fume hood. The reduction of protein disulfides with 2-mercaptoethanol proceeds rapidly via a two-step process involving an intermediate mixed disulfide (Fig. 68). Due to its strong reducing properties, the reagent is used most often when complete disulfide reduction is required. It also can be used to cleave disulfide-containing cross-linking agents. Usually a concentration of 0.1 *M* 2-mercaptoethanol will cleave a disulfide-containing cross-linker and liberate conjugated proteins (Chapter 7, Section 1).



2-ME;
2-Mercaptoethanol
MW 78.13

2-Mercaptoethanol is used as a reducing additive in a number of biochemical reagents. It is used as a reductant for a Gram-negative bacteria lysis buffer (Schwinghamer, 1980; Scopes, 1982), as the second-dimensional equilibration buffer for 2D electrophoresis (Dunbar, 1987), as the sample reducing buffer for SDS–polyacrylamide gel electrophoresis (Laemmli, 1970), and as a participant in the *o*-phthalaldehyde reaction for the detection of primary amines (Jones and Gilligan, 1983).

Protocol for Preparation and Use of a Gram-Negative Bacteria Lysis Buffer

1. Prepare a solution consisting of 2.5 ml glycerol, 100 µl of 10% Triton X-100 (Pierce Surfact-Amps X-100), and 10 µl 2-mercaptoethanol.
2. Add 10 g of wet packed cells to the lysis buffer and stir vigorously for 30 min.
3. Add 30 ml of an extraction buffer consisting of 20 m*M* potassium phosphate, pH 7, 1 m*M* EDTA, 0.2 mg/ml lysozyme, and 10 µg/ml DNase I.
4. Add 5 mg PMSF dissolved in 0.5 ml acetone and 0.1 mg pepstatin A.
5. Centrifuge for 20 min at 15,000 *g*. Recover the extracted, solubilized material in the supernatant.

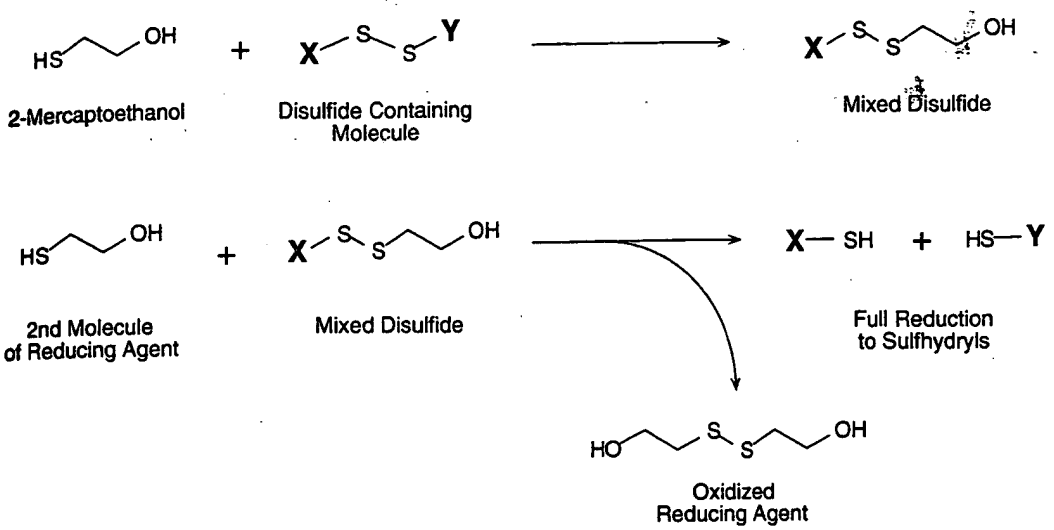


Figure 68 The reduction of disulfides by 2-mercaptoethanol proceeds through a mixed disulfide intermediate.

Protocol for Preparation and Use of the Second-Dimension Equilibration Buffer for 2D Gels

The following procedure relates to electrophoretic protocols where the first dimension is developed by isoelectric focusing (in tube gels) and the second dimension is a size exclusion separation by SDS–polyacrylamide electrophoresis in a slab gel.

1. Add 4 g SDS and 20 ml of 10% glycerol to 150 ml of 0.125 *M* Tris, pH 6.8, and adjust the final volume to 200 ml. Once dissolved, add a few crystals of bromophenol blue, mix, and pass the solution through a 0.2-μm filter. For storage, freeze in 10- to 15-ml aliquots.
2. Immediately before use, add 2-mercaptoethanol to a final concentration of 0.5–0.8%.
3. Incubate the first dimensional electrophoresis tube gel in this reducing buffer for 15 min. Drain off excess buffer and electrophorese in the second dimension.

SDS Sample Buffer for Running Electrophoresis Size Separations under Reducing Conditions

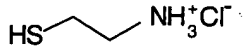
1. Dissolve 2 g of SDS, 0.75 g Tris base, and 10 ml of glycerol in 90 ml of water. Adjust the pH to 6.8 and bring the final volume to 100 ml.
2. To a small aliquot of the above buffer, add 2-mercaptoethanol to obtain a final concentration of 2–5%. Only 200 μl of this buffer typically is required to treat and reduce about 10–500 μg of protein. Solubilize the protein sample in this buffer.
3. Incubate a sealed tube at 95°C for 5–10 min or in a boiling bath for 1–2 min. Electrophorese immediately.

o-Phthalaldehyde Solution for the Fluorescent Detection of Primary Amines (see Section 4.3, OPA)

1. Add 3 ml of the detergent Brij-35 (as a 30% solution) and 2 ml of 2-mercaptoethanol to 950 ml of fluoraldehyde reagent diluent (all reagents from Pierce).
2. Dissolve 0.5–0.8 g of *o*-phthalaldehyde crystals in about 10 ml of methanol.
3. Mix the OPA solution with the solution from (1) and store under nitrogen in sealed glass bottles at 4°C. The addition of an aliquot of this solution to a sample containing primary amines will yield an intense blue fluorescence.

2-Mercaptoethylamine

2-Mercaptoethylamine is a disulfide reducing agent that has found widespread application in the partial reduction of immunoglobulin molecules. The reagent is supplied as a solid in the hydrochloride form (Pierce) and possesses very little of the sulfhydryl odor of 2-mercaptoethanol. When used under nondenaturing conditions, 2-mercaptoethylamine can cleave, almost selectively, the disulfide bonds between the heavy chains of IgG. This directed reduction is important for generating sulfhydryls while preserving antigen binding activity.



2-MEA;
2-Mercaptoethylamine
Hydrochloride
MW 113.62

The complex structure of an antibody molecule creates two antigen binding sites from the interaction of the hypervariable regions on both the heavy and the light chains. For this reason, heavy–light chain pairing must remain intact during any modification procedure to ensure that antigen binding activity is retained. In addition, it is important that any chemistry take place away from the antigen binding sites so they are not sterically blocked by modification reagents or by subsequent conjugation steps. 2-Mercaptoethylamine can be used to cleave disulfides primarily in the hinge region of IgG—away from the antigen binding sites—thus preserving the disulfides that hold the heavy and light chains together (Yoshitake *et al.*, 1979). It also can be used to reduce $F(ab')_2$ fragments, because they still retain the hinge region disulfides of intact IgG (Fig. 69).

Once reduced with 2-mercaptoethylamine, immunoglobulins will be cleaved in half, forming two heavy chain–light chain molecules of MW 75,000–80,000 and each containing one antigen binding site. These half molecules of IgG will possess reactive sulfhydryls in the hinge region that can be used in conjugation protocols with sulfhydryl-reactive cross-linking reagents. For instance, a reduced antibody may be used to make a conjugate with a maleimide-activated enzyme, forming a reagent useful in immunoassays (Chapter 10, Section 1.1). Similarly, $F(ab')_2$ fragments may be reduced to yield two molecules, each containing an antigen binding site. Making conjugates with this low-molecular-weight fragment can dramatically reduce background in assay systems or provide access to antigens restricted to higher-molecular-weight con-

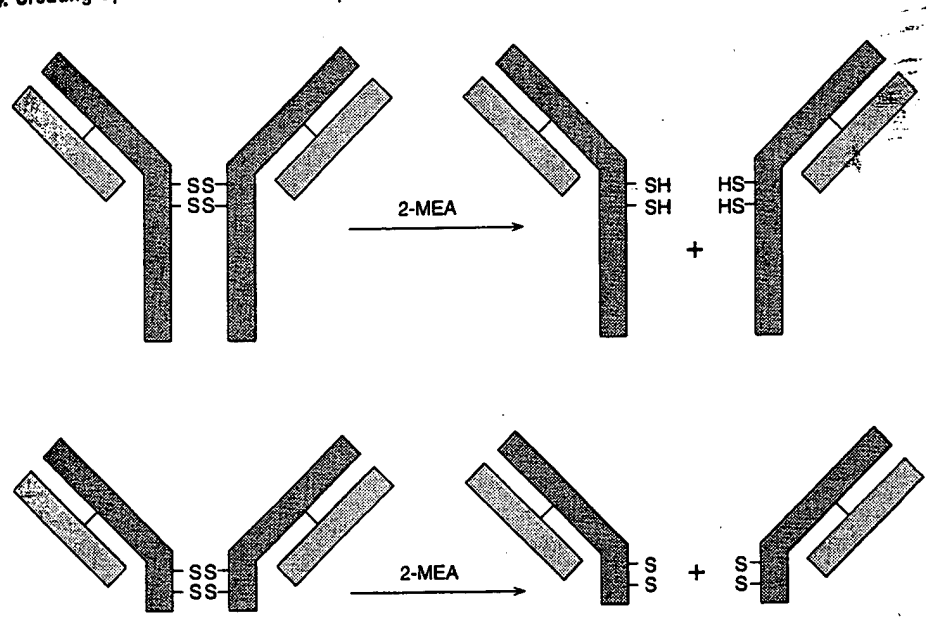


Figure 69 Disulfide reducing agents such as 2-mercaptoethylamine can be used to cleave the disulfide bonds in the hinge region of antibody molecules. Either intact IgG molecules or $F(ab')_2$ fragments may be reduced in this manner to yield monofunctional antigen binding fragments.

jugates made with intact antibody (such as in immunohistochemical staining techniques).

Protocol

1. Dissolve the antibody to be reduced at a concentration of 10 mg/ml in 20 m*M* sodium phosphate, 0.15 *M* NaCl, pH 7.4, containing 1–10 m*M* EDTA.
2. To each milliliter of the antibody solution, add 6 mg of 2-mercaptoethylamine hydrochloride (final concentration is 50 m*M*). Mix to dissolve.
3. Incubate the solution in a sealed tube for 90 min at 37°C.
4. Purify the reduced IgG from excess 2-mercaptoethylamine and reaction byproducts by dialysis or gel filtration using Sephadex G-25. All buffers should contain 1–10 m*M* EDTA to preserve the free sulfhydryls from metal-catalyzed oxidation. The sulfhydryl containing half-antibody may now be used in conjugation protocols that use —SH-reactive heterobifunctional cross-linkers (Chapter 10, Section 1.1).

TCEP

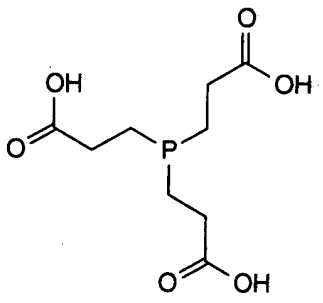
The reduction of disulfide bonds with trivalent phosphines has been known for some time (Ruegg and Rudingder, 1977; Kirley, 1989; Levison *et al.*, 1969). Unfortunately, trialkylphosphines generally are water-insoluble, undergo autoxidation, and are extremely odious.

The water-soluble tris(2-carboxyethyl)phosphine (TCEP) was synthesized and used to cleave rapidly organic disulfides to sulfhydryls in water (Burns *et al.*, 1991). The

advantage of using this phosphine derivative in disulfide reduction as opposed to previous ones is its excellent stability in aqueous solution, its lack of reactivity with other common functionalities, and its freedom from odor.

The reaction of TCEP with biological disulfides proceeds with initial cleavage of the S—S bond followed by oxidation of the phosphine (Fig. 70). The stability of the phosphine oxide bond that is formed in this process is great enough to prevent reversal of the reaction. Since this reaction is performed without any added —SH compounds, subsequent conjugation with the generated sulfhydryl groups can be done without removal of excess TCEP or reaction by-products (provided the conjugation step does not involve disulfide exchange reactions, such as with the active disulfide-containing reagent SPDP; Chapter 5, Section 1.1).

Although TCEP is capable of rapidly and quantitatively reducing simple organic disulfides in solution, it requires the presence of a deforming agent to reduce fully all disulfides in proteins. Without opening up the internal disulfides in many protein molecules, TCEP will not be able to reduce them. For complete reduction of IgG, it was found that 20 m*M* TCEP and 5 min of boiling was needed (Hines, 1992). Partial reduction, however, is possible of some more accessible disulfides in aqueous buffers at room temperature.



TCEP
Tris(2-carboxyethyl)phosphine
(hydrochloride)
MW 250.19

Protocol for the Complete Reduction of Disulfide Bonds within Protein Molecules

1. Dissolve the protein to be reduced at a concentration of 1–10 mg/ml in 20 m*M* sodium phosphate, 0.15 *M* NaCl, pH 7.4. Other buffers and pH values also may be used. A strong denaturant may be added (6 *M* guanidine or 8 *M* urea) to this solution to promote protein unfolding and make buried disulfides more accessible.
2. Add TCEP to a final concentration of 20 m*M*.
3. Place in a sealed tube and incubate in a boiling water-bath for 5 min. If a denaturant was included in the buffer from (1), then high temperature may not be necessary. Alternatively, incubate the sample at 50°C for 30 min.
4. To remove excess TCEP and reaction by-products, dialyze the solution or purify by gel filtration using a buffer containing 1–10 m*M* EDTA.

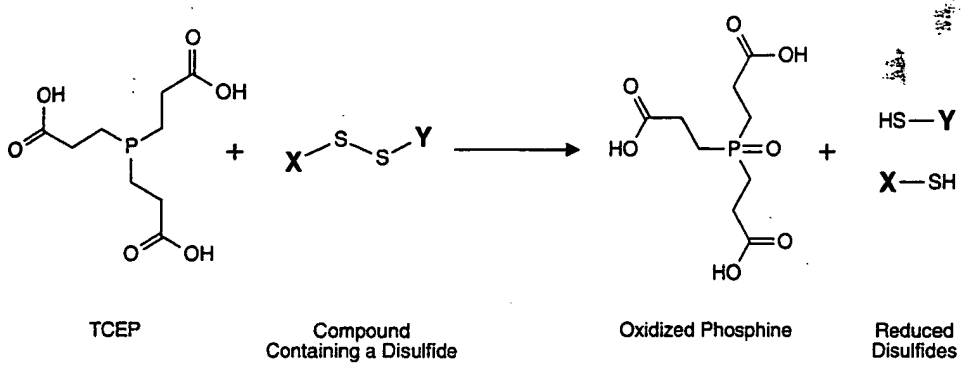


Figure 70 TCEP reduction of disulfides proceeds without the use of thiol compounds.

Protocol for Partial Reduction of Protein Disulfides or for Cleaving Disulfide-Containing Modification Reagents

1. Dissolve the protein to be reduced at a concentration of 1–10 mg/ml in 20 m*M* sodium phosphate, 0.15 *M* NaCl, pH 7.4. Other buffers and pH values also may be used. Do not add a denaturant to unfold protein structure.
2. Add TCEP to a final concentration of 20 m*M*.
3. Incubate for 2 h at room temperature.
4. To remove excess TCEP and reaction by-products, dialyze the solution or purify the protein by gel filtration using a buffer containing 1–10 m*M* EDTA.

Immobilized Disulfide Reductants

Many extracellular proteins like immunoglobulins, protein hormones, serum albumin, pepsin, trypsin, ribonuclease, and others contain one or more indigenous disulfide bonds. For functional and structural studies of proteins, it is often necessary to cleave these disulfide bridges. Disulfide bonds in proteins are commonly reduced with small, soluble mercaptans, such as 2-mercaptoethanol, thioglycolic acid, and cysteine. High concentrations of mercaptans (molar excess of 20- to 1000-fold) are usually required to drive the reduction to completion.

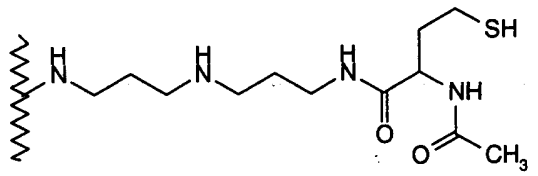
Cleland (1964) showed that dithiothreitol (DTT) and dithioerythritol (DTE) are superior reagents in reducing disulfide bonds in proteins (Section 4.1). DTT and DTE have low oxidation–reduction potential and are capable of reducing protein disulfides at concentrations far below that required with 2-mercaptoethanol. However, even these reagents must be used in an approximately 20-fold molar excess in order to get close to 100% reduction of a protein.

An immobilized disulfide reductant usually consists of an insoluble beaded support material such as agarose that has been modified with a small ligand containing a terminal sulfhydryl group. The presence of densely coupled sulfhydryl groups on the matrix creates enormous disulfide reducing potential. Simply mixing a solution of a disulfide-containing peptide or protein with the immobilized reductant efficiently breaks any disulfide linkages and creates free sulfhydryls. This is done without extraneous sulfhydryl contamination by the reductant, as in the case of soluble reductants.

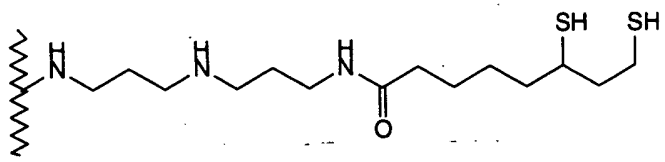
The use of immobilized disulfide reductants thus have the following advantages over solution phase agents:

1. Immobilized disulfide reductants can be used to reduce all types of biological disulfides without liberating product or by-product contaminants.
2. Soluble components that interfere with the assay of free thiol groups are not present if immobilized disulfide reductants are used.
3. Small molecules containing disulfide bonds (such as cystine-containing peptides) may be reduced and isolated simply by removing the immobilized reductant. Separation of reduced molecules from reductant is much more difficult if a soluble reducing agent is used with low-molecular-weight disulfides.
4. Immobilized disulfide reductants can be easily regenerated and reused many times.

Immobilized dihydrolipoamide (thioctic acid) (Gorecki and Patchornick, 1973, 1975) and immobilized *N*-acetyl-homocysteine thiolactone (Eldjarn and Jellum, 1963; Jellum, 1964) are the two most commonly used immobilized disulfide reductants. The author, along with Krishna Mallia of Pierce Chemical, has successfully used immobilized reductants to reduce many types of biological disulfides, including small molecules like oxidized glutathione and bovine insulin.



Immobilized N-Acetyl homocysteine
attached to a diaminodipropylamine spacer



Immobilized Dihydrolipoamide
attached to a diaminodipropylamine spacer

Immobilized disulfide reductants may be synthesized as described in Hermanson *et al.* (1992) or obtained commercially (Pierce).

Reduction of Peptides Using Immobilized Reductants

Note: For optimal reduction of peptides, the following steps should be performed at room temperature.

1. Pack an immobilized reductant gel (2 ml settled gel) in a disposable polypropylene column and wash with 5 ml of 0.1 *M* sodium phosphate buffer, pH 8, containing 1 m*M* EDTA (equilibration buffer).

2. Prepare the sulfhydryl column by washing with a disulfide reducing agent. Apply 10 ml of freshly made 10 m*M* DTT solution (15.4 mg of DTT dissolved in 10 ml of equilibration buffer). This treatment converts the immobilized ligands into a fully reduced form (free —SH groups).
3. Wash the column with 20 ml of equilibration buffer 1 to remove free DTT.
4. Apply to the column 1 ml of peptide solution (dissolved in equilibration buffer) to be reduced. Normally, small peptides (molecular weight less than or equal to that of insulin) require no deforming agent (denaturant) such as guanidine to be completely reduced.
5. After the sample has completely entered into the gel bed, wash the column with 9 ml of equilibration buffer, while collecting 1-ml fractions.
6. Monitor the elution of reduced peptide from the column by measuring the absorbance at 280 nm (if peptide absorbs at this wavelength) as well as by performing an Ellman's assay (Section 4.1) for sulfhydryl groups using a small aliquot (10–20 µl) of each collected fraction.
7. Regenerate the sulfhydryl containing support by following steps 2 and 3 above. Such columns can be regenerated and reused at least 10 times without any significant decrease in the reductive capacity.
8. Store the column in 0.02% sodium azide at 4°C.

Reduction of Proteins Using Immobilized Reductants

Note: For optimal reduction of proteins, the following steps must be performed at room temperature.

1. Pack an immobilized reductant gel (2 ml) in a disposable polypropylene column and wash with 5 ml of 0.1 *M* sodium phosphate buffer, pH 8, containing 1 m*M* EDTA (equilibration buffer 1).
2. Prepare the sulfhydryl column by washing with a disulfide reducing agent. Apply 10 ml of freshly made 10 m*M* DTT solution (15.4 mg of DTT dissolved in 10 ml of equilibration buffer 1).
3. Wash the column with 10 ml of equilibration buffer 1 and 10 ml of 0.1 *M* sodium phosphate buffer, pH 8, containing 1 m*M* EDTA and 6 *M* guanidine hydrochloride (equilibration buffer 2) to remove free DTT.
4. Apply to the column 1 ml of protein solution (dissolved in equilibration buffer 2) to be reduced. The inclusion of a denaturant in the solution deforms the protein structure so that inner disulfides are available to the immobilized reductant. Without the presence of guanidine or another deforming agent (i.e., urea, SDS), only partial reduction of the protein is possible.
5. After the sample has completely entered the gel bed, incubate the column at room temperature for 1 h.
6. Wash the column with 9 ml of equilibration buffer 2 while 2-ml fractions are collected.
7. Monitor elution of reduced protein from the column by measuring the absorbance at 280 nm as well as by performing an Ellman's assay for sulfhydryl groups (Section 4.1) using a small aliquot (50–100 µl) of each collected fraction.
8. Regenerate the sulfhydryl containing column by following steps 2 and 3 above.

Such columns can be regenerated and reused at least 10 times without any significant decrease in the reductive capacity.

9. Store the column in 0.02% sodium azide at 4°C.

Sodium Borohydride

Perhaps the simplest route to the reduction of disulfide groups in peptides is the use of sodium borohydride ($NaBH_4$). This common reducing agent often used in organic synthesis is able to reduce specifically disulfides to free thiols without affecting any of the other major functional groups in proteins. Gailit (1993) developed a protocol for borohydride reduction that avoids any purification steps to remove the reducing agent after the reaction. Thus, peptides reduced by this protocol can be used immediately in bioconjugate applications without additional steps.

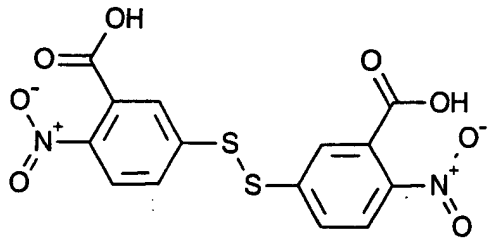
Protocol

1. Dissolve the peptide to be reduced in a buffer at pH 8–10. Sodium phosphate or sodium bicarbonate at 0.1 *M* work well.
2. Add sodium borohydride (Aldrich) to the peptide solution to obtain a final concentration of 0.1 *M*. Generation of hydrogen bubbles will occur as the borohydride is dissolved.
3. Incubate at room temperature for 30–60 min.
4. Adjust the pH of the reaction to pH 4 using dilute HCl. Incubate for 10 min to ensure the complete destruction of excess borohydride. Hydrogen bubbles again will be evolved from the solution.
5. Readjust the pH to the optimal value for the bioconjugate application to be done. Use the reduced peptide immediately to prevent reoxidation of the thiols to disulfides.

Ellman's Assay for the Determination of Sulfhydryls

Ellman's reagent, 5,5′-dithio*bis*(2-nitrobenzoic acid), reacts with sulfhydryls under slightly alkaline conditions to release the highly chromogenic compound, 5-thio-2-nitrobenzoic acid (TNB) (Ellman, 1959; Riddles *et al.*, 1979) (Fig. 71). The reagent contains a disulfide bond between two TNB groups and reacts with free sulfhydryls to create a mixed disulfide product. The target of the reaction is the unprotonated, conjugate base form of the thiol R—S^-. At pH 8, the release of one TNB group per available thiol provides a yellow-colored product with an extinction coefficient at 412 nm of 13,600 $M^{-1}cm^{-1}$. The increase in absorbance at this wavelength is directly proportional to the concentration of sulfhydryls in solution. Correlation to a standard curve of known sulfhydryl concentrations allows accurate measurement of the thiol content in unknown samples.

Ellman's reagent has been used not only for the determination of sulfhydryls in proteins and other molecules, but also as a precolumn derivatization reagent for the separation of thiol compounds by HPLC (Kuwata *et al.*, 1982), in the study of thiol-dependent enzymes (Masamune *et al.*, 1989; Tsukamoto and Wakil, 1988; Alvear *et al.*, 1989), and to create sulfhydryl-reactive chromatography supports for the coupling of affinity ligands (Jayabaskaran *et al.*, 1987). Another important use of the compound



Ellman's Reagent
5,5'-Dithio-bis-(2-nitrobenzoic acid)
MW 396.4

is in the assessment of conjugation procedures using sulfhydryl-reactive cross-linking agents (Chapter 9, Section 5).

Depending on the conditions, an Ellman's assay can detect as little as 10 n*M* cysteine concentration. The linearity can extend into the m*M* range, making the test extremely flexible for different sample situations.

Protocol

1. Dissolve Ellman's reagent (Pierce) in 0.1 *M* sodium phosphate, pH 8, at a concentration of 4 mg/ml.
2. Prepare a set of standards by dissolving cysteine in 0.1 *M* sodium phosphate, pH 8, at an initial concentration of 2 m*M* (3.5 mg/ml) and serially diluting this solution (1:1) with reaction buffer down to at least 0.125 m*M*. This will produce five solutions of cysteine for generating a standard curve. If a more dilute concentration range is required, continue to dilute serially until a set of standards in the desired range is obtained.
3. Label a set of test tubes according to the standards and samples to be used. Add 250 µl of each standard and sample to the appropriate tubes. If the samples are in a buffer that may significantly change the pH of the reaction buffer, the samples should be buffer-exchanged or dialyzed into 0.1 *M* sodium phosphate, pH 8, before running the assay.
4. Add 50 µl of Ellman's reagent to each standard and sample tube. Mix well.
5. Incubate at room temperature for 15 min.
6. Measure the absorbance of each solution at 412 nm.
7. Plot the absorbance versus cysteine concentration for each of the standards.

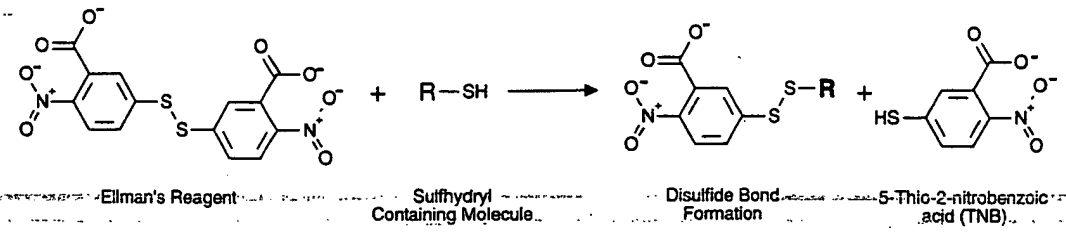


Figure 71 The reaction of Ellman's reagent with a sulfhydryl group releases the chromogenic TNB anion, which can be quantified by its absorbance at 412 nm.

Determine the sulfhydryl concentration of the samples by comparison to the standard curve.

4.2. Introduction of Carboxylate Groups

Modification of various functional groups in macromolecules with the following types of reagents will introduce carboxylate functions for further derivatization purposes. Amines, sulfhydryls, and histidine and methionine side chains are readily modified to contain short molecules terminating in a carboxylic acid. The short chain can serve as a spacer to enhance steric accommodations and the terminal carboxylate group can facilitate subsequent couplings with amines or hydrazides. The introduction of carboxylates also affects the overall charge characteristics or pI of the molecule being derivatized. The modification of amine residues by acylation with anhydrides not only eliminates the positive charge contribution of the protonated amine, but also adds the negative charge contribution of the acid. The result may be a change of minus two in net charge per group modified. While the reactions involved in such derivatizations are conducted under relatively mild conditions, severe alterations in net charge may cause some macromolecules, like proteins, to denature or lose activity. In addition, if the group being modified happens to be critical for active center operation then the functional group may be compromised regardless of conditions. While the following reactions are facile and efficient, it should be kept in mind that in certain instances modification may lead to inactivity.

Modification of Amines with Anhydrides

Acid anhydrides, as their name implies, are formed from the dehydration reaction of two carboxylic acid groups (Fig. 72). Anhydrides are highly reactive toward nucleophiles and are able to acylate a number of the important functional groups of proteins and other macromolecules. Upon nucleophilic attack, the anhydride yields one carboxylic acid for every acylated product. If the anhydride was formed from monocarboxylic acids, such as acetic anhydride, then the acylation occurs with release of one carboxylate group. However for dicarboxylic acid anhydrides, such as succinic anhydride, upon reaction with a nucleophile the ring structure of the anhydride opens, forming the acylated product modified to contain a newly formed carboxylate group.

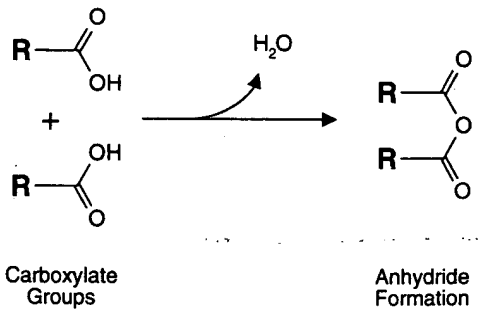


Figure 72 Anhydrides are created from two carboxylate groups by the removal of one molecule of water.

Thus, anhydride reagents may be used both to block functional groups and to convert an existing functional group into a carboxylic acid.

Protein functional groups able to react with anhydrides include the α-amines at the N-terminals, the ε-amine of lysine side chains, cysteine sulfhydryl groups, the phenolate ion of tyrosine residues, and the imidazolyl ring of histidines. However, acylation of cysteine, tyrosine, and histidine side chains forms unstable complexes that are easily reversible to regenerate the original group. Only amine functional groups of proteins are stable to acylation with anhydride reagents (Fraenkel-Conrat, 1959; Smyth, 1967).

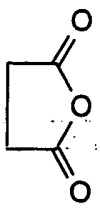
Another potential site of reactivity for anhydrides in protein molecules is modification of any attached carbohydrate chains. In addition to amino group modification in the polypeptide chain, glycoproteins may be modified at their polysaccharide hydroxyl groups to form esterified derivatives. Esterification of carbohydrates by acetic anhydride, especially cellulose, is a major industrial application for this compound. In aqueous solutions, however, esterification will be a minor product, since the oxygen of water is about as strong a nucleophile as the hydroxyls of sugar residues.

The major side reaction to the desired acylation product is hydrolysis of the anhydride. In aqueous solutions anhydrides may break down by the addition of one molecule of water to yield two unreactive carboxylate groups. The presence of an excess of the anhydride in the reaction medium usually is enough to minimize the effects of competing hydrolysis.

Since both hydrolysis and acylation yield the release of carboxylic acid functional groups, the medium becomes acidic during the course of the reaction. This requires either the presence of a strongly buffered environment to maintain the pH or periodic monitoring and adjustment of the pH with base as the reaction progresses.

Succinic Anhydride

Succinic acid is a four-carbon molecule with carboxylic acid groups on both ends. The anhydride has a five-atom cyclic structure that is highly reactive toward nucleophiles, especially amines. Attack of a nucleophile at one of the carbonyl groups opens the anhydride ring, forming a covalent bond with that carbonyl and releasing the other to create a free carboxylic acid (Klotz, 1967). Succinylation of positively charged amino groups of proteins and other molecules thus creates amide bond derivatives and converts the cationic site into a negatively charged carboxylate (Fig. 73). Succinylated proteins often experience dramatic changes in their three-dimensional structure. Subunits may dissociate (Klotz and Keresztes-Nagy, 1962), enzymatic activity may be compromised (Riordan and Valle, 1963, 1964), and the molecular radius and viscosity



Succinic Anhydride
MW 100

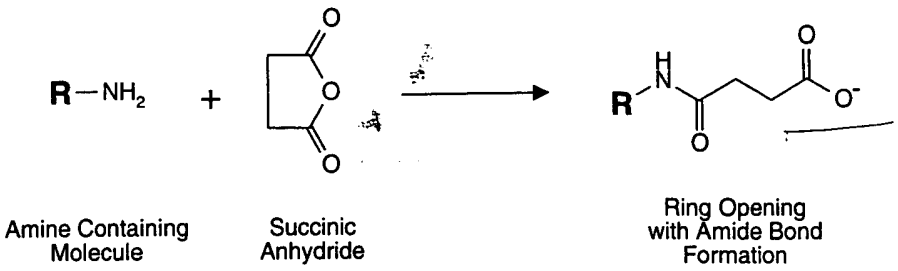


Figure 73 Succinic anhydride reacts with primary amine groups in a ring-opening process, creating an amide bond and forming a terminal carboxylate.

may be increased (Habeeb *et al.*, 1958). Other effects on protein conformation and function have been studied as well (Meighen *et al.*, 1971; Shetty and Rao, 1978; Shiao *et al.*, 1972).

Succinic anhydride also may react with protein phenolate side chains of tyrosine residues and the —OH group of aliphatic hydroxy amino acids (Fig. 74). The phenolate ester derivatives are unstable above pH 5, whereas the serine and threonine esters are relatively stable but may be specifically cleaved by treatment with hydroxylamine (Gounaris and Perlman, 1967).

A succinylated casein derivative that has nearly all its amines blocked can be used as a substrate in protease assays (Hatakeyama *et al.*, 1992). As the casein is degraded by a protease, free amines are created from α-chain cleavage and release of α-amino groups. The creation of amines can be monitored by an amine detection reagent such as trinitrobenzene sulfonic acid (TNBS; Section 4.3). The procedure forms the basis for a highly sensitive assay for protease activity.

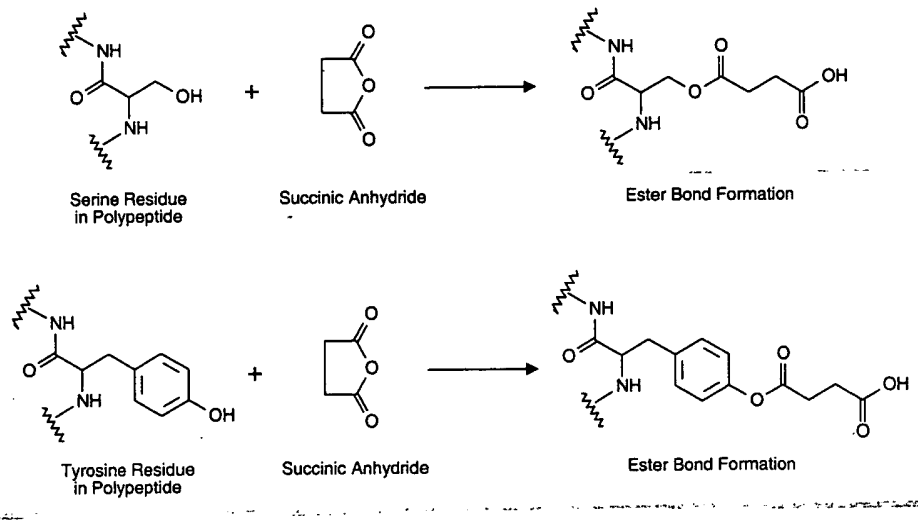


Figure 74 The hydroxyl group of serine residues and the phenolate ring of tyrosine groups may be modified with succinic anhydride to produce relatively unstable ester bonds. In aqueous conditions these reactions are minor due to competing hydrolysis by water.

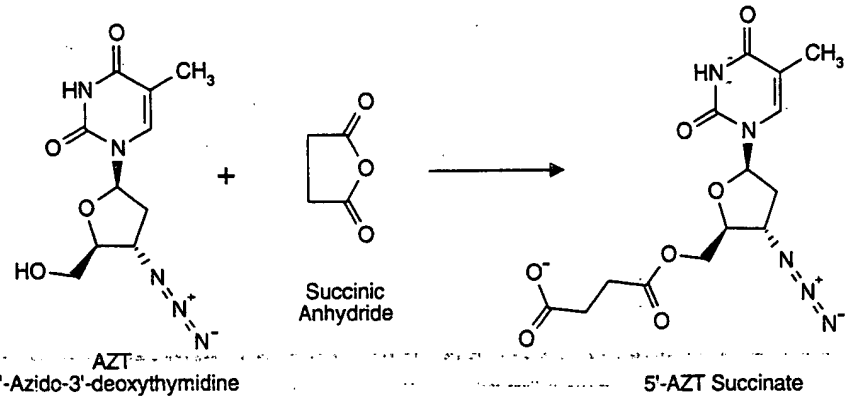
Succinylated derivatives of nucleic acids may be prepared by reaction of the anhydride with available —OH groups. The reaction forms relatively stable ester derivatives that create carboxylates on the nucleotide for further conjugation or modification (Fig. 75). This method has been used in nucleic acid synthesis (Matteucci and Caruthers, 1980) and to derivatize nucleotide analogs such as AZT (Tadayoni *et al.*, 1993).

Succinic anhydride also is a convenient extender for creating spacer arms on chromatography supports. Supports derivatized with amine-terminal spacers may be succinylated to block totally the amine functional groups and form terminal carboxylic acid linkers for coupling amine-containing affinity ligands (Cuatrecasas, 1970).

Molecules modified with succinic anhydride to create terminal carboxylate functional groups may be further conjugated to amine-containing molecules by use of amide bond-forming reagents such as carbodiimides (Chapter 3, Section 1).

Protocol

1. Dissolve (or suspend in the case of insoluble polymers or support materials) the amine-containing molecule to be succinylated in a buffer having a pH between 6 and 9. Higher pH buffers will cause the reaction to occur faster and result in more amines in an unprotonated state. Suitable buffer salts include sodium acetate, sodium phosphate, and sodium carbonate in a 0.1–1.0 *M* concentration. Avoid buffers containing primary amine groups such as Tris. Alternatively, the substance may be dissolved in water and the pH maintained in the proper range by periodic addition of NaOH. This is conveniently done by means of a pH stat. Even in buffered reactions, the pH should be monitored to prevent severe acidification of the reaction solution, which could damage the molecule being modified.
2. Add a quantity of succinic anhydride to the reaction medium to provide at least a 5–10 molar excess of reagent over the amount of amines to be modified. Even greater molar excesses may be required for total blocking of all the amines of some proteins. When adding solid succinic anhydride, multiple additions may



AZT
3'-Azido-3'-deoxythymidine

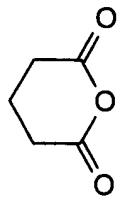
Figure 75 Succinic anhydride has been used in nonaqueous conditions to modify the 5′-hydroxyl group of nucleic acid derivatives such as AZT.

be done to maintain solubility of the reagent in the reaction solution. The anhydride also may be dissolved in dry dioxane before addition to aid in dissolution.

3. React for at least 1–2 h at room temperature. To ensure complete blocking of all amine groups, the reaction may be continued overnight.
4. Remove excess reactants from the succinylated molecule by dialysis, gel filtration, or some other suitable method. The efficiency of amine modification may be assessed by use of the TNBS test for amines (Section 4.3). A negative test for amines indicates complete succinylation.

Glutaric Anhydride

Glutaric acid is a linear, five-carbon molecule with carboxylic acid groups on both ends. It contains one additional carbon in length than the similar compound succinic acid. The anhydride of glutaric acid forms a cyclic structure containing six atoms. Attack of a nucleophile, such as an amino group, on one of the carbonyl groups of glutaric anhydride opens the ring, forming an amide linkage and liberating the other carboxylic acid (Fig. 76). Reaction with the phenolate of tyrosine or the sulfhydryl group of cysteine forms unstable linkages (an ester and a thioester, respectively) that can easily hydrolyze. As with succinic anhydride, however, aliphatic hydroxyl groups such as those of serine and threonine may be modified with glutaric anhydride to create more stable ester bonds (see above).



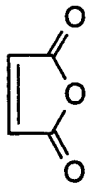
Glutaric Anhydride
MW 114

Protocol

The procedure for the modification of amine-containing compounds with glutaric anhydride is identical to that described for succinic anhydride, above.

Maleic Anhydride

Maleic acid is a linear four-carbon molecule with carboxylate groups on either end, similar to succinic acid, but with a double bond between the central carbon atoms. The anhydride of maleic acid is a cyclic molecule containing five atoms in its ring. Although the reactivity of maleic anhydride is similar other such reagents like succinic anhydride, the products of maleylation are much more unstable toward hydrolysis, and the site of unsaturation lends itself to additional side reactions. Acylation products of amino groups with maleic anhydride are stable at neutral pH and above, but they readily hydrolyze at acid pH values (around pH 3.5) (Butler *et al.*, 1967). Maleylation of sulfhydryls and the phenolate of tyrosine are even more sensitive to hydrolysis.



Maleic Anhydride
MW 98

As with other cyclic anhydrides, the acylation of an amine residue proceeds with elimination of the potential positive charge of the amine and addition of the negative charge created by the anhydride ring opening (Fig. 77). Thus, a molecule can undergo a change of minus two in net charge per site of maleylation. Proteins extensively modified with maleic anhydride may spontaneously dissociate into subunits or experience a general opening of their three-dimensional structures (Sia and Horecker, 1968; Uyeda, 1969).

The double bond of maleic anhydride may undergo free radical polymerization with the proper initiator. Polymers of maleic anhydride (or copolymers made with another monomer) are commercially available (Polysciences). They consist of a linear hydrocarbon backbone (formed from the polymerization of the vinyl groups) with cyclic anhydrides protruding from the chain. Such polymers are highly reactive toward amine-containing molecules.

Maleic acid imides (maleimides) are derivatives of the reaction of maleic anhydride and ammonia or primary amine compounds. The double bond of maleimides may undergo an alkylation reaction with sulfhydryl groups to form a stable thioether bond (Chapter 2, Section 2.2). Maleic anhydride may presumably undergo the same irreversible reaction with cysteine residues and other sulfhydryl compounds.

Proteins derivatized with maleic anhydride exhibit an increase in their absorptivity at wavelengths below 280 nm, likely due to the addition of the unsaturated carbon—carbon bond. The extent of maleylation may be estimated by measuring the absorbance increase before and after modification (Freedman *et al.*, 1968).

Protocol

Modification of amines with maleic anhydride is done essentially the same as that described for succinic anhydride (this section), except the pH of the reaction should be kept alkaline (pH 8–9) at all times to prevent unwanted deacylation. Deblocking of

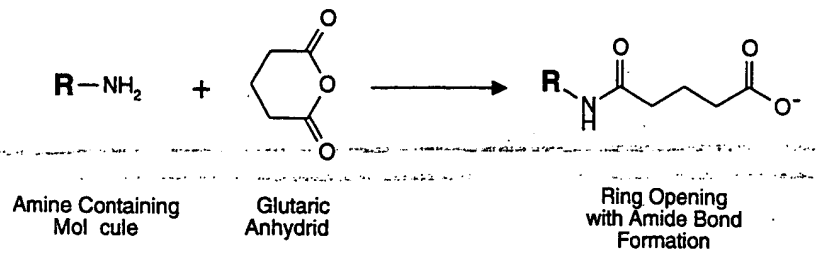


Figure 76 Glutaric anhydride reacts with amines in a ring-opening process to create an amide bond linkage and a terminal carboxylate group.

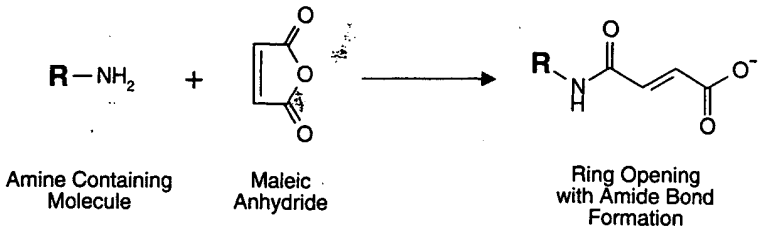


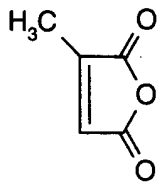
Figure 77 Maleic anhydride reacts with amine groups in a ring-opening process to create carboxylate derivatives.

maleylated amines can be accomplished according to the following procedure of Butler *et al.* (1967).

1. Adjust the pH of the maleylated protein or other molecule to pH 3.5 with formic acid and aqueous NH_3.
2. Incubate the solution at 37°C for 30 h.
3. Stop the deblocking reaction by the addition of NaOH to raise the pH back to neutrality.

Citraconic Anhydride

Citraconic anhydride (or 2-methylmaleic anhydride) is a derivative of maleic anhydride that is even more reversible after acylation than maleylated compounds. At alkaline pH values (pH 7–8) the reagent effectively reacts with amine groups to form amide linkages and a terminal carboxylate. However, at acid pH (3–4), these bonds rapidly hydrolyze to release citraconic acid and free the amine (Fig. 78) (Dixon and Perham, 1968; Klapper and Klotz, 1972; Habeeb and Atassi, 1970; Shetty and Kinsella, 1980). Thus, citraconic anhydride has been used to block temporarily amine groups while other parts of a molecule are undergoing derivatization. Once the modification is complete, the amines then can be unblocked to create the original structure.



Citraconic Anhydride MW 113

Acid labile, heterobifunctional cross-linking reagents have been synthesized using 2-methylmaleic anhydride at one end (Blattler *et al.*, 1985a,b). Amines can be reacted with the anhydride end under alkaline conditions to form amide linkages. The other end, containing another functional group, in this case a maleimide group, is then made to react with a sulfhydryl-containing molecule. After the conjugation is complete, the citraconylamide end can be specifically released by lowering the pH.

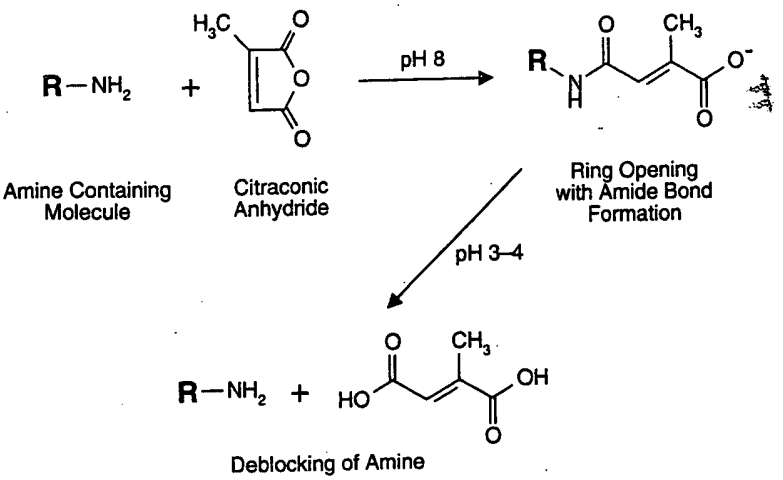


Figure 78 Citraconic anhydride can be used to block amine groups reversibly. The amide bond derivative is unstable to acidic conditions.

Protocol

1. Dissolve the amine-containing molecule to be modified in a buffer having a pH between 8 and 9. Maintenance of a high pH is necessary due to the high tendency of citraconylamides to hydrolyze at lower pH values. Suitable buffer salts include sodium phosphate and sodium carbonate in a 0.1–1.0 *M* concentration. Avoid buffers containing primary amine groups such as Tris. Alternatively, the substance may be dissolved in water and the pH maintained in the proper range by periodic addition of NaOH. This is conveniently done by means of a pH stat.
2. Add a quantity of citraconic anhydride to the reaction medium to provide at least a 5–10 molar excess of reagent over the amount of amines to be modified. Even greater molar excesses may be required for total blocking of all the amines of some proteins. When adding citraconic anhydride, multiple additions may be done to maintain solubility of the reagent in the reaction solution.
3. React for at least 1–2 h at room temperature. To ensure complete blocking of all amine groups, the reaction may be continued overnight.
4. Remove excess reactants from the citraconylated molecule by dialysis, gel filtration, or some other suitable method. The efficiency of amine modification may be assessed by use of the TNBS test for amines (Section 4.3). A negative test for amines indicates complete modification.

To remove the citraconic modifications and free the amine groups, the protein may be treated in one of two ways:

1. Adjust the pH of the citraconylated molecule to 3.5–4.0 by addition of acid. Incubate at room temperature overnight or for at least 3 h at 30°C.

or

2. Treat the citraconylated molecule with 1 *M* hydroxylamine at pH 10 for 3 h at room temperature.

Modification of Sulfhydryls with Iodoacetate

Iodoacetate (and bromoacetate) can react with a number of functional groups within proteins: the sulfhydryl group of cysteine, both imidazolyl side chain nitrogens of histidine, the thioether of methionine, and the primary ε-amine group of lysine residues and N-terminal α-amines (Gurd, 1967). The relative rate of reaction with each of these residues is generally dependent on the degree of ionization and thus the pH at which the modification is done. The exception to this is methioninyl thioethers that react rapidly at nearly all pH values above about 1.7 (Vithayathil and Richards, 1960). The reaction products of these groups with iodoacetate are illustrated in Fig. 79. The only reaction resulting in one definitive product is that of the alkylation of cysteine sulfhydryls, giving the carboxymethylcysteinyl derivative (Cole *et al.*, 1958). Histidine groups may be modified at either nitrogen atom of its imidazolyl side chain. Both monosubstituted derivatives and disubstituted products of the imidazole ring are possible (Crestfield *et al.*, 1963). With primary amine groups such as in the side chain of lysine residues, the products of the reaction are either the secondary amine, mono-

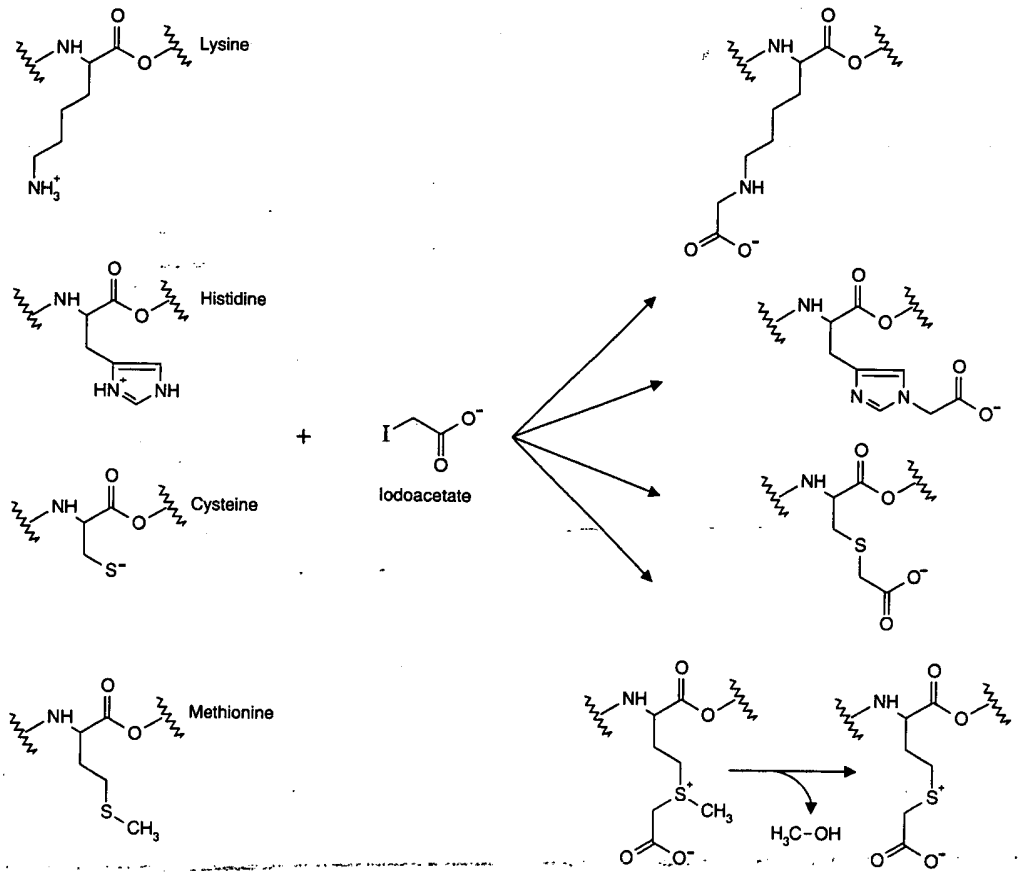
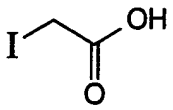


Figure 79 Iodoacetate can modify a number of amino acid side chains in proteins, forming alkylated derivatives containing a terminal carboxylate.

carboxymethyllysine, or the tertiary amine derivative, dicarboxymethyllysine. Methionine thioether groups give the most complicated products, some of which rearrange or decompose unpredictably. The only stable derivative of methionine is where the terminal methyl group is lost to form carboxymethylhomocysteine, the same product as that in the reaction of iodoacetate with homocysteine.



Iodoacetate
MW 185.9

The relative reactivity of α-haloacetates toward protein functionalities is sulfhydryl > imidazolyl > thioether > amine. Among halo derivatives the relative reactivity is I > Br > Cl > F, with fluorine being almost unreactive. The α-haloacetamides have the same trend of relative reactivities, but will obviously not create a carboxylate functional group. The acetamide derivatives typically are used only as blocking reagents.

Thus, iodoacetate has the highest reactivity toward sulfhydryl cysteine residues and may be directed specifically for —SH modification. If iodoacetate is present in limiting quantities (relative to the number of sulfhydryl groups present) and at slightly alkaline pH, cysteine modification will be the exclusive reaction. The specificity of this modification has been used in the design of heterobifunctional cross-linking reagents, where one end of the cross-linker contains an iodoacetamide derivative and the other end contains a different functional group directed at another chemical target (see SIAB, Chapter 5, Section 1.5).

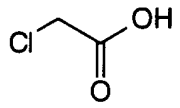
Protocol

1. Dissolve the sulfhydryl-containing protein or macromolecule to be modified at a concentration of 1–10 mg/ml in 50 m*M* Tris, 0.15 *M* NaCl, 5 m*M* EDTA, pH 8.5. EDTA is present to prevent metal-catalyzed oxidation of sulfhydryl groups. The presence of Tris, an amine containing buffer, should not affect the efficiency of sulfhydryl modification. Not only do amines generally react slower than sulfhydryls, the amine in Tris buffer is of particularly low reactivity. If Tris does pose a problem, however, use 0.1 *M* sodium phosphate, 0.15 *M* NaCl, 5 m*M* EDTA, pH 8.
2. Add iodacetate to a concentration of 50 m*M* in the reaction solution. Alternatively, add a quantity of iodoacetate representing a 10-fold molar excess relative to the number of —SH groups present. An estimation of the sulfhydryl content in the protein to be modified can be accomplished by performing an Ellman's assay (Section 4.1). Readjust the pH if necessary. To aid in adding a small quantity of iodoacetic acid to the reaction, a concentrated stock solution may be made in the reaction buffer, the pH readjusted, and an aliquot added to the protein solution to give the desired concentration.
3. Mix and react for 2 h at room temperature.
4. Purify the modified protein from excess iodoacetate by dialysis or gel filtration.
5. An Ellman's assay comparing the unmodified protein to the iodoacetylated protein may be done to assess the degree of modification.

Modification of Hydroxyls with Chloroacetic Acid

Chloroacetic acid can be used to transform a rather unreactive hydroxyl into a carboxylate group that can be used in a variety of conjugation reactions. The reaction proceeds under basic conditions, yielding a stable ether bond terminating in a carboxymethyl group (Fig. 80) (Plotz and Rifai, 1982; Brunswick *et al.*, 1988). Side reactions will occur with other nucleophiles, such as amines, if they are present in the molecule to be modified. The reagent is used most often to modify pure polysaccharides or hydroxyl-containing polymers that contain no other functional groups.

The following protocol illustrates the modification of a dextran polymer with chloroacetic acid.



Chloroacetic Acid
MW 94.47

Protocol

1. In a fume hood, prepare a solution consisting of 1 *M* chloroacetic acid in 3 *M* NaOH.
2. Immediately add dextran polymer to a final concentration of 40 mg/ml. Mix well to dissolve.
3. React for 70 min at room temperature with stirring.
4. Stop the reaction by adding 4 mg/ml of solid NaH_2PO_4 and adjusting the pH to neutral with 6 *N* HCl.
5. Remove excess reactants by dialysis.

4.3. Introduction of Primary Amine Groups

Primary amine groups on proteins consisting of N-terminal α-amines and lysine side-chain ε-amines are typically present in abundant quantities for modification or conjugation reactions. Occasionally, however, a protein or peptide will not contain sufficient amounts of available amines to allow for an efficient degree of coupling to another molecule or protein. For instance, horseradish peroxidase (HRP), a popular enzyme to employ in the preparation of antibody conjugates, only possesses two free amines that

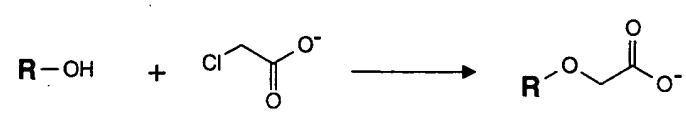


Figure 80 Chloroacetic acid can be used to create a carboxylate group from a hydroxyl.

can participate in conjugation protocols. Creating additional amines on HRP allows for higher amounts of modification and thus produces more active conjugates.

Other nonprotein molecules, such as nucleic acids and oligonucleotides, may not normally possess primary amines of sufficient nucleophilicity to react with common modification reagents. The ability to add amine functional groups to these molecules is sometimes the only route to successful conjugation. Creating amines at specific sites within these molecules allows for site-directed modification at known positions, thus better ensuring active conjugates once formed.

The following reagents and techniques can be used to transform directly carboxylates or sulfhydryl groups into reactive amine functional groups. In addition, sugars, polysaccharides, or carbohydrate-containing macromolecules may be modified to contain amines after mild periodate activation to form aldehyde groups.

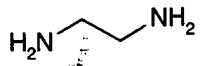
Modification of Carboxylates with Diamines

Carboxylic acids may be covalently modified with short compounds containing primary amines at either end to form amide linkages. The result of such alterations is to block the carboxylates and form terminal amino groups. Reacting the diamine in excess ensures that only one end of the compound couples to each carboxylate and does not cross-link the molecule being modified. Amide bond formation may be accomplished by several methods including carbodiimide mediated coupling (Chapter 2, Section 1.11), active ester intermediates such as *N*-hydroxysuccinimide esters (Chapter 2, Section 1.4), and the use of carbonylating compounds like *N,N'*-carbonyldiimidazole (Chapter 2, Section 3.2). A combination of the water-soluble carbodiimide EDC and sulfo-NHS also is an efficient way of creating amide linkages (Chapter 2, Section 1.11).

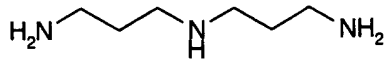
Diamines that can be used for aminoalkylation include ethylene diamine, 1,3-diaminopropane, 3,3'-imino*bis*propylamine (also known as diaminodipropylamine), 1,6-diaminohexane, and the short-chain Jeffamine derivative EDR-148 containing a hydrophilic, polyether, 10-atom chain (Texaco Chemical Co.). Ethylene diamine is perhaps the most popular choice for protein carboxylate modification. Its short chain length ensures minimal steric effects and virtually no hydrophobic interactions. Diaminodipropylamine provides a longer spacer arm and has been used extensively as a bridging molecule for coupling carboxylate containing ligands to insoluble supports (Hermanson *et al.*, 1992). The long hydrocarbon chain of 1,6-diaminohexane, however, may induce hydrophobic effects and probably should be avoided. The longest diamine of the group is the Jeffamine compound. Its chain is extremely hydrophilic and should function as an excellent modifier of carboxylates when a longer spacer is desired.

Diamine modification of proteins can have dramatic effects on the net charge of the molecule, usually significantly raising the pI from the native state. The amide linkage eliminates the negative potential of the carboxylate and the terminal amine adds a positive charge. Thus, diamine modification has a net effect of changing the overall charge by plus two for every carboxylate residue coupled. Heavily modified proteins may exhibit vital changes in activity due to the alteration of microenvironmental charge at each site of modification. In some cases, native conformation may be changed and activity completely lost.

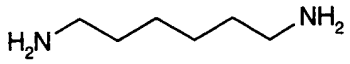
Raising the pI of macromolecules also can significantly alter the immune response



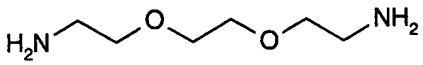
Ethylenediamine
MW 60



3,3'-Iminobispropylamine
(diaminodipropylamine)
MW 131



1,6-Diaminohexane
MW 116



Jeffamine EDR-148
MW 148

toward them on *in vivo* administration. Cationized proteins (those modified with diamines to increase their net charge or pI) are known to generate an increased immune response compared to their native forms (Muckerheide *et al.*, 1987a; Domen *et al.*, 1987; Apple *et al.*, 1988). The use of cationized BSA as a carrier protein for hapten conjugation can result in a dramatically higher antibody response toward a coupled hapten (Chapter 9, Section 2.1).

The following protocol using the carbodiimide EDC is an efficient way of modifying protein carboxylates with diamines either to increase the amount of amines present for further conjugation or to create a cationized protein having an increased net charge (Fig. 81). Note that glycoproteins containing sialic acid may be modified at this sugar's —COOH group in addition to coupling at C-terminal, aspartic acid, and glutamic acid functions on the polypeptide chain. Other carboxylate containing macromolecules may be modified using this procedure as well.

Protocol

1. Dissolve the protein to be modified at a concentration of 1–10 mg/ml in 0.1 *M* MES, pH 4.7 (coupling buffer). Other buffers may be used as long as they do not contain groups that can participate in the carbodiimide reaction. Avoid carboxylate- or amine-containing buffers such as citrate, acetate, glycine, or Tris. Higher pH conditions may be used up to about pH 7.5 (in sodium phosphate buffer) without severely affecting the yield of modification. The protein in solid form also may be added directly to the diamine solution prepared in (2).
2. Dissolve the diamine chosen for modification at a concentration of 1 *M* made up in the coupling buffer. If a free-base form of diamine is used, then the solution will become highly alkaline on dissolution. This operation also will generate

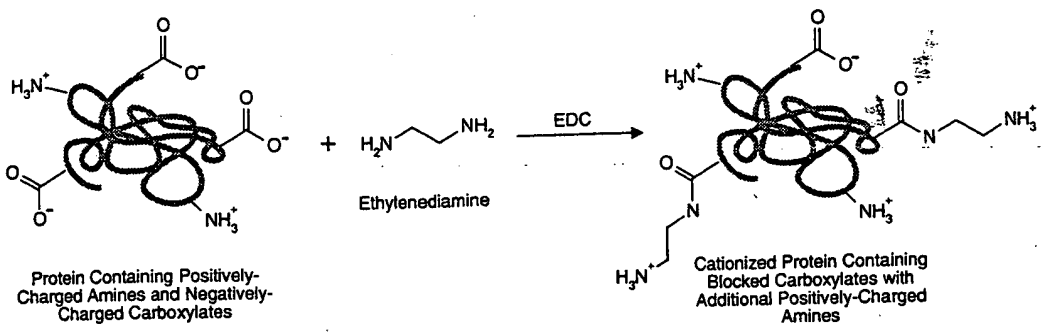


Figure 81 Cationization of protein molecules can be done using ethylene diamine to modify carboxylate groups using a carbodiimide reaction process.

heat—the solution process being highly exothermic. The easiest way to dissolve such a diamine is to add initially the correct amount to a beaker containing a quantity of crushed ice equal to the final solution volume desired. The ice should be made from deionized water or the equivalent to maintain purity. All operations should be done in a fume hood. Next, add an equivalent weight of concentrated HCl and mix. As the mixing becomes complete, the ice will almost totally melt and provide nearly the correct final solution volume. Finally, add an amount of MES buffer salt to bring its concentration to 0.1 *M* and adjust the solution pH to 4.7. In some cases, the dihydrochloride form of the diamine is commercially available and can be used to avoid such unpleasant pH adjustments. For instance, ethylenediamine dihydrochloride is available from Aldrich. It can be added to the 0.1 *M* MES buffer without a significant change in pH

3. Add the protein solution to an equal volume of diamine solution and mix. Alternatively, the solid protein can be dissolved directly in the diamine solution at the indicated concentration.
4. Add EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; Pierce) to a final concentration of 2 mg/ml in the reaction solution. To aid in the addition of a small amount of EDC, a higher concentration stock solution may be prepared in water and an aliquot added to the reaction to give the proper concentration. Since EDC is labile in aqueous solutions, the stock solution must be made quickly and used immediately.
5. React for 1–2 h at room temperature.
6. Purify the modified protein by extensive dialysis against 0.02 *M* sodium phosphate, 0.15 *M* NaCl, pH 7.4 (PBS), or another suitable buffer.

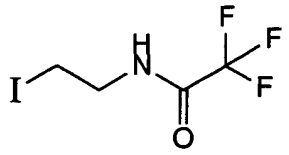
The changes that occur in the pI of a protein modified with diamines may be assessed by isoelectric focusing or by general electrophoresis based on relative migration due to charge. A cationized protein will possess a higher pI value or migrate further toward the anode than its native form. Using the above protocol typically alters the net charge of bovine serum albumin from a native pI of 4.9 to the highly basic range of pI 9.5 to over pI 11.

Modification of carboxylate groups with diamines also may be done in organic solvent for those molecules insoluble in aqueous buffers. Some peptides are quite soluble in solvents such as DMF and DMSO, but relatively insoluble in water. Such

molecules may be reacted in these solvents using the carbodiimide DCC using the same basic reactant ratios as given above for EDC in aqueous solutions (Chapter 3, Section 1.4).

Modification of Sulfhydryls with N-(β-Iodoethyl)trifluoroacetamide (Aminoethyl-8)

The conversion of sulfhydryl groups on cysteine residues or other molecules to amine-containing groups may be accomplished by aminoethylation with *N*-(β-iodoethyl) trifluoroacetamide (Schwartz *et al.*, 1980). The haloalkyl group specifically reacts with sulfhydryls to form the aminoalkyl derivative in one step. Under the conditions of the reaction, the trifluoroacetate amine-protecting group spontaneously hydrolyzes to expose the free primary amine without the need for a secondary deblocking step (Fig. 82). This reagent is commercially available from Pierce Chemical under the name Aminoethyl-8.



Aminoethyl–8™ Reagent
N-(iodoethyl)trifluoroacetamide
MW 267

Aminoethyl-8 has an advantage over ethylenimine modification (next section), due to the potential polymerization of ethylenimine in aqueous solutions. Such polymers are highly cationic and may nonspecifically block protein. The specificity of Aminoethyl-8 for sulfhydryls makes it an optimum choice for modification.

For small molecules containing sulfhydryls or for low-molecular-weight peptides containing cysteine residues, modification may proceed without deforming agents. However, for intact proteins containing both disulfides and free sulfhydryls, a denaturant and a disulfide reducing agent may be required to open buried or structurally inaccessible groups if complete modification is desired.

Protocol

1. Dissolve the protein to be aminoalkylated at a concentration of 1–10 mg/ml in 6 *M* guanidine hydrochloride, 0.2 *M* *N*-ethylmorpholine acetate, pH 8.1. All water used in preparing buffers should be deoxygenated by boiling followed by cooling and bubbling with nitrogen. Small molecules that do not require denaturants to expose internal disulfides or sulfhydryls may be modified without using guanidine treatment.
2. Add dithiothreitol (DTT) to obtain a 20-fold molar excess over the amount of disulfides present.
3. React for 4 h at room temperature, maintaining a blanket of nitrogen over the solution.
4. Adjust the pH to 8.6 with NaOH, and heat the solution to 50°C.

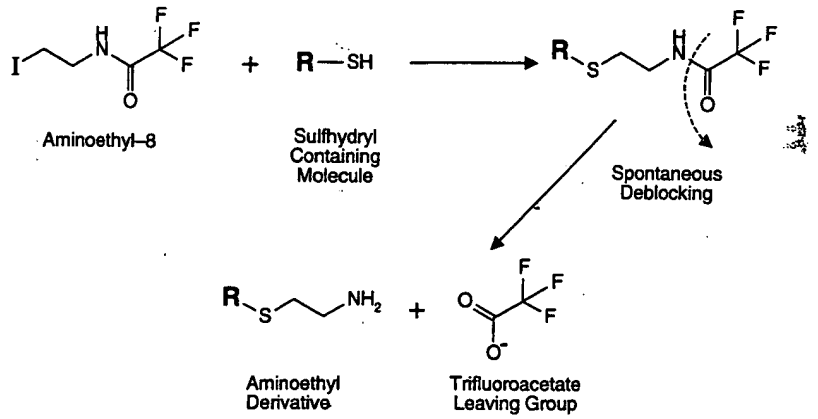


Figure 82 Aminoethyl-8 can be used to transform a sulfhydryl group into an amine. The intermediate spontaneously undergoes deblocking to release the primary amine group.

5. Add a quantity of Aminoethyl-8 in methanol to equal a 25-fold molar excess over the amount of sulfhydryl present (including the amount of DTT added). The solution in methanol should be made concentrated enough so only a small amount of methanol must be added to the reaction solution (i.e., no more than 10% of the final volume). A second addition of modifying agent may be made after 1 h to drive the reaction more completely toward total —SH aminoalkylation.
6. React for 3 h at 50°C.
7. Purify the modified protein or other macromolecule by gel filtration or dialysis. Occasionally, complete modification with Aminoethyl-8 will cause precipitation of the protein.

Modification of Sulfhydryls with Ethylenimine

The cyclic compound ethylenimine reacts with protein sulfhydryl groups causing ring opening and forming the aminoalkyl derivative, *S*-(2-aminoethyl)cysteine (Raftery and Cole, 1963, 1966) (Fig. 83). Under physiological conditions ethylenimine is virtually specific for sulfhydryls with no cross-reactivity toward other protein functional groups. At acid pH, a small degree of reactivity occurs with methionine residues, forming *S*-(2-aminoethyl)methionine sulfonium ion (Schroeder *et al.*, 1967). Since aminoethylated cysteine groups resemble the side-chain structure of lysine residues, except for the replacement of one methylene group with a thioether, these modifications make them susceptible to tryptic hydrolysis, although at an abbreviated rate (Plapp *et al.*, 1967; Wang and Carpenter, 1968).

Ethylenimine
MW 43

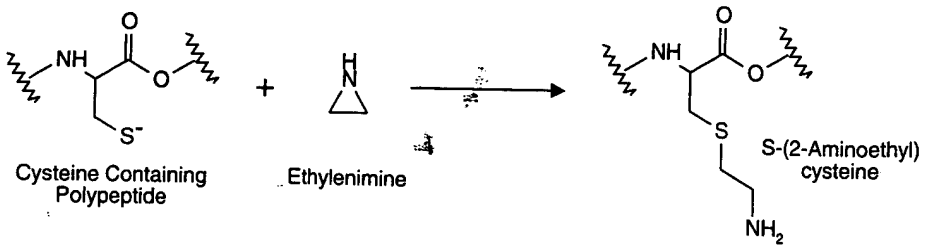


Figure 83 The small compound ethylenimine can react with sulfhydryls to form aminoethyl derivatives.

Ethylenimine may be used to introduce additional sites of tryptic cleavage for protein structural studies. In this case, complete sulfhydryl modification is usually desired. Proteins are treated with ethylenimine under denaturing conditions (6–8 *M* guanidine hydrochloride) in the presence of a disulfide reductant to reduce any disulfide bonds before modification. Ethylenimine may be added directly to the reducing solution in excess (similar to the procedure for Aminoethyl-8 described previously) to totally modify the —SH groups formed.

The disadvantage of using ethylenimine for protein modification stems from the fact that in the presence of water, slow formation of polyethylenimine occurs. The polymer is highly positively charged at physiological pH and can interact strongly with protein molecules, masking sites of potential sulfhydryl modification. Also, the polymer may have terminal aziridine residues (Chapter 2, Section 2.3), making it reactive and potentially forming a covalent attachment with the protein (Dermer and Ham, 1969).

Modification of Sulfhydryls with 2-Bromoethylamine

2-Bromoethylamine may undergo two reaction pathways in its modification of sulfhydryl groups in proteins (Fig. 84). In the first scheme, the thiolate anion of cysteine attacks the No. 2 carbon of 2-bromoethylamine to release the halogen and form a thioether bond (Lindley, 1956). This straightforward reaction mechanism is similar to the modification of sulfhydryls with iodoacetate (Section 4.2). In a two-step, secondary

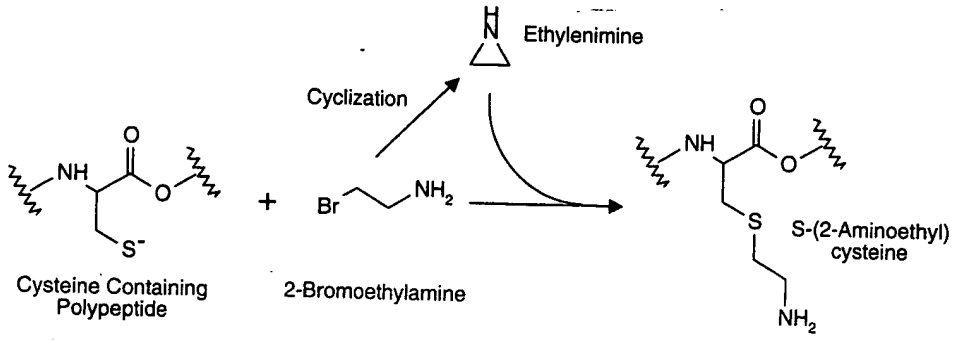
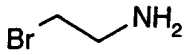


Figure 84 2-Bromoethylamine can be used to transform a thiol into an amine. The reaction may proceed through the intermediate formation of ethylenimine, yielding an aminoethyl derivative.

process, 2-bromoethylamine is converted under alkaline conditions to the cyclic ethylenimine derivative by the intramolecular attack of its primary amine on the number 2 carbon, causing release of the halogen and ring formation (Cole, 1967). Ethylenimine then goes on to react with the sulfhydryl to form the aminoalkylated derivative (as described in the previous section). The two-step reaction is slower than direct aminoalkylation by either 2-bromoethylamine or ethylenimine.



2-Bromoethylamine
MW 123.92

Protocol

1. Dissolve the protein or peptide to be aminoalkylated at cysteine sulfhydryls in 0.5 *M* sodium carbonate. If cystine disulfides are present, add a 10- to 25-fold molar excess of DTT to reduce them fully to free sulfhydryls.
2. Add a quantity of 2-bromoethylamine to obtain a 10-fold molar excess over the number of sulfhydryls present in the sample, including any added DTT.
3. React overnight at room temperature.
4. Purify the modified protein by gel filtration or dialysis.

Modification of Carbohydrates with Diamines

Carbohydrates or oligosaccharides may be modified to contain primary amino groups by selective reaction with a diamine compound. Several reaction pathways may be used to accomplish this modification. In some cases, a particular carbohydrate may contain sugar residues that possess potential amine coupling groups without prior derivatization to form such functional groups. For example, if carboxylate containing sugars are present like sialic or uronic acid (Fig. 85), then direct modification with a diamine is possible using the carbodiimide coupling protocol described previously in this section.

If carboxylates are lacking in the carbohydrate molecule, then indigenous hy-

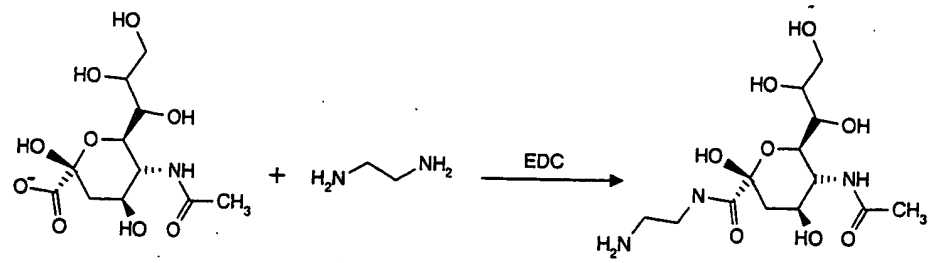


Figure 85 Carboxylate-containing sugars may be modified with diamines using a carbodiimide-mediated reaction to create available amine groups for subsequent conjugation.

droxyls may be utilized to create aldehydes for coupling diamines by one of two routes. The simplest method of creating amine reactive groups in sugar molecules is by oxidation using sodium periodate (Section 4.4). Periodic acid cleaves adjacent hydroxyls to form highly reactive aldehyde groups (Rothfus and Smith, 1963). At a concentration of 1 m*M* sodium periodate specifically cleaves only at the adjacent hydroxyls between the No. 7, 8, and 9 carbon atoms of sialic acid residues (Van Lenten and Ashwell, 1971; Wilchek and Bayer, 1987). The product is the formation of one aldehyde group on the No. 7 carbon and liberation of two molecules of formaldehyde. The sialic acid aldehyde then can be coupled with diamines by Schiff base formation and reductive amination (Chapter 2, Section 5.3 and Chapter 3, Section 4).

Oxidation of polysaccharides using 10 m*M* or greater concentrations of sodium periodate results in the cleavage of adjacent diol containing carbon—carbon bonds on other sugars besides just sialic acid residues. Glycoproteins and polysaccharides may be modified using this procedure to form multiple formyl functional groups for coupling diamines or other amine containing molecules.

In some instances, reducing sugars are present that can be reductively aminated without prior periodate treatment. A reducing end of a monosaccharide, a disaccharide, or a polysaccharide chain may be coupled to a diamine by reductive amination to yield an aminoalkyl derivative bound by a secondary amine linkage (Fig. 86).

An alternative to the use of chemical means to create formyl groups is the specific modification afforded by sugar oxidasaes (Section 4.4). For instance, galactose oxidase may be reacted with a carbohydrate containing terminal D-galactose or *N*-acetyl-D-galactosamine residues to transform the C-6 hydroxyl group into an aldehyde (Avigad *et al.*, 1962). Subsequent reaction with a diamine yields the desired amine modification.

The appropriate protocols for diamine modification of various carbohydrate or glycoprotein derivatives may be found in the indicated sections.

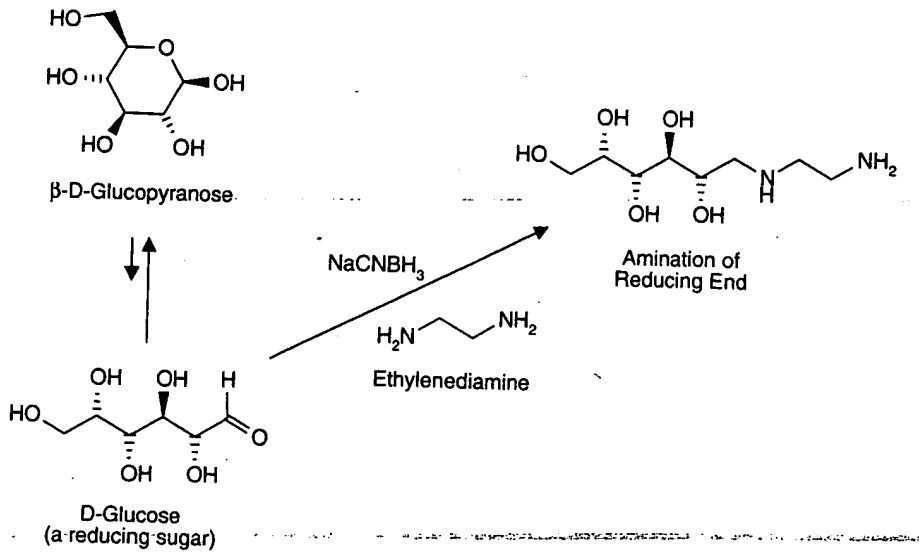


Figure 86 Reducing sugars may be aminated with diamines in the presence of sodium cyanoborohydride to produce amine modifications.

Modification of Alkylphosphates with Diamines

Alkylphosphate groups can be made to react with diamines to form aminoalkylphosphoramidate modifications. The primary amine thus formed then may be used to conjugate with other molecules containing amine reactive groups. In this sense, DNA or RNA may be modified with a diamine at the 5′ phosphate group mediated by a carbodiimide reaction. N-substituted carbodiimides can react with phosphate groups to form highly reactive phosphodiester derivatives that are extremely short-lived in aqueous solution (Chapter 3, Section 1) (Fig. 87). This active species then can react with a nucleophile such as a primary amine to form a phosphoramidate bond (Chu *et al.*, 1986). The process is analogous to the activation of a carboxylate by a carbodiimide with subsequent coupling to an amine-containing molecule to form an amide linkage (Williams and Ibrahim, 1981).

In most procedures, the water-soluble carbodiimide EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) is the most effective mediator of this reaction. Both EDC and its reaction by-products are fully soluble in aqueous buffers and can be easily separated from the modified aminoalkylphosphate (Chapter 3, Section 1.1).

In some methods, the reaction is carried out in a two-step process by first forming an intermediate, reactive phosphorylimidazolide by EDC conjugation in an imidazole buffer. Next, the diamine, in this case cystamine, is reacted with the activated oligonucleotide, causing the imidazole to be replaced by the amine and creating a phosphoramidate linkage (Chu *et al.*, 1986). An easier protocol was described by Ghosh *et al.*, (1990) in which the oligo, cystamine, and EDC were all reacted together in an imidazole buffer. A modification of this method developed by Zanocco *et al.* (1993) is described in Chapter 17, Section 2.1.

Modification of Aldehydes with Ammonia or Diamines

Aldehyde groups can be converted into terminal amines by a reductive amination process with ammonia or a diamine compound. The reaction proceeds by initial formation of a Schiff base interaction—a dehydration step yielding an imine derivative. Reduction of the Schiff base with sodium cyanoborohydride or sodium borohydride produces the primary amine (in the case of ammonia) or a secondary amine derivative terminating in a primary amine (for a diamine compound) (Fig. 88).

This simple strategy can be used to add amine residues to polysaccharide molecules after formation of aldehydes by periodate or enzymatic oxidation (Section 4.4). Thus,

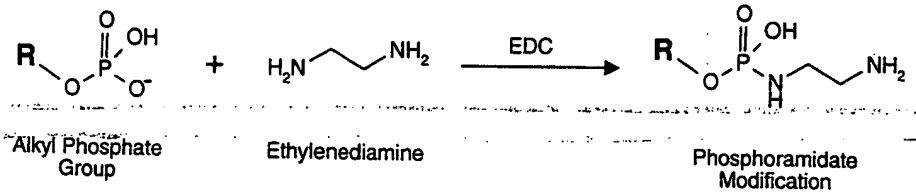


Figure 87 Phosphate groups may be modified to possess amines by a carbodiimide reaction in the presence of a diamine.

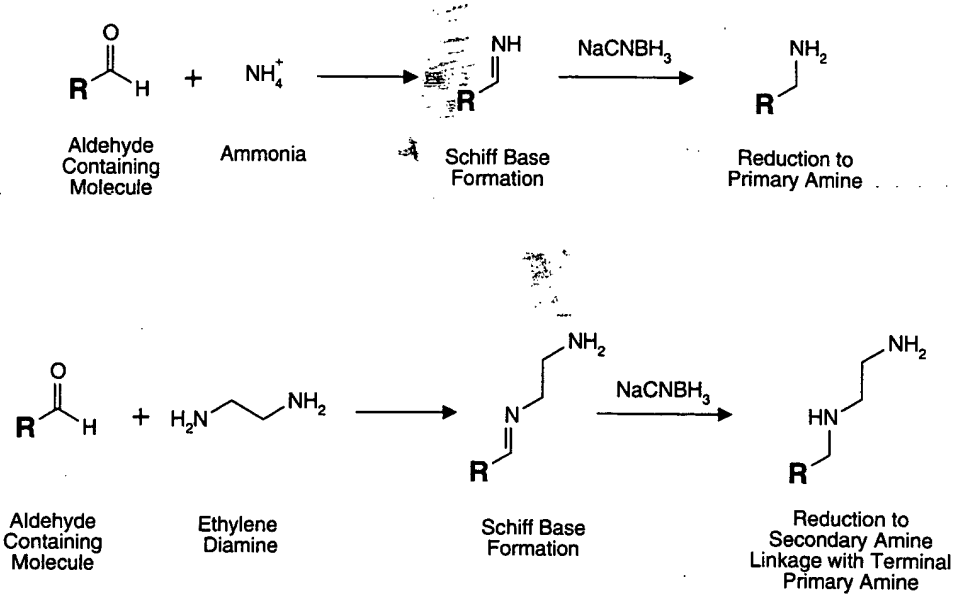


Figure 88 Aldehydes may be transformed into primary amines by reaction with ammonia or a diamine in the presence of a reducing agent.

glycoconjugates or carbohydrate polymers such as dextran may be derivatized to contain amines for further conjugation reactions.

The reaction occurs rapidly at alkaline pH (7–10), with higher pH values resulting in better yields due to faster Schiff base formation. To ensure complete conversion of available aldehydes to amines, add the ammonia or diamine compound to the reaction in at least a 10-fold molar excess over the expected number of formyl groups present, Diamines that are commonly used for this process include ethylene diamine, diaminodipropylamine (3,3′-iminobispropylamine), 1,6-diaminohexane, and the Jeffamine derivative EDR-148 containing a hydrophilic, 10-atom chain (Texaco Chemical Co.).

Introduction of Arylamines on Phenolic Compounds

Compounds having phenol ring structures, such as tyrosine residues in proteins, often can be derivatized to contain aromatic amine groups through a two-stage reaction process. First, the phenolic ring is nitrated with tetranitromethane in aqueous solution to add a nitro group *ortho* (or *para*, if available) to the hydroxyl. This type of modification can be used to detect tyrosine residues by the strong absorptivity of the unprotonated (at pH 9), 3-nitrophenolate ring at 428 nm (extinction coefficient = 4200 $M^{-1}cm^{-1}$) (Sokolovsky *et al.*, 1967). The method has been used to quantify the tyrosine content in porcine trypsinogens and trypsins and to modify a variety of other proteins (Vincent *et al.*, 1970; Lundbald, 1991).

The nitrophenol group also may be reduced to an aminophenyl derivative in alkaline conditions with the use of sodium dithionite ($Na_2S_2O_4$). The amine then can be used to conjugate with an amine-reactive cross-linking reagent to label peptides or proteins at their tyrosine side chains. In addition, this strategy can be a route to creating modifiable amine groups on aromatic molecules other than just tyrosine. For

instance, the Bolton–Hunter reagent (Chapter 8, Section 4.5) can be used to modify amine groups on proteins, leaving a phenolic end that is typically used as a site for radioiodination. However, such a derivative also could be used to create an arylamine for further transformation into a highly reactive diazonium group for coupling to tyrosines or phenolic functional groups in other molecules (Fig. 89) (Chapter 2, Section 6.1).

Protocol

1. Dissolve the protein-containing tyrosine residues (or another phenolic macromolecule) in 0.02 *M* sodium phosphate, 0.15 *M* NaCl, pH 7.4, at a concentration of 2–4 mg/ml.
2. With stirring, add to each milliliter of the protein solution, 20 µl of 0.15 *M* tetranitromethane in 95% ethanol (Sigma). Make the addition in small aliquots if more than several milliliters of solution are to be derivatized.
3. React for 1 h at room temperature.
4. Quench the reaction by immediate gel filtration using a column of Sephadex G-25 (Pharmacia). Equilibrate the column and perform the chromatography using 0.2 *M* sodium borate, pH 9, so that the protein will be at the proper pH for the reduction step. After the separation, a determination of the modification level may be done by measuring its absorbance at 428 nm.

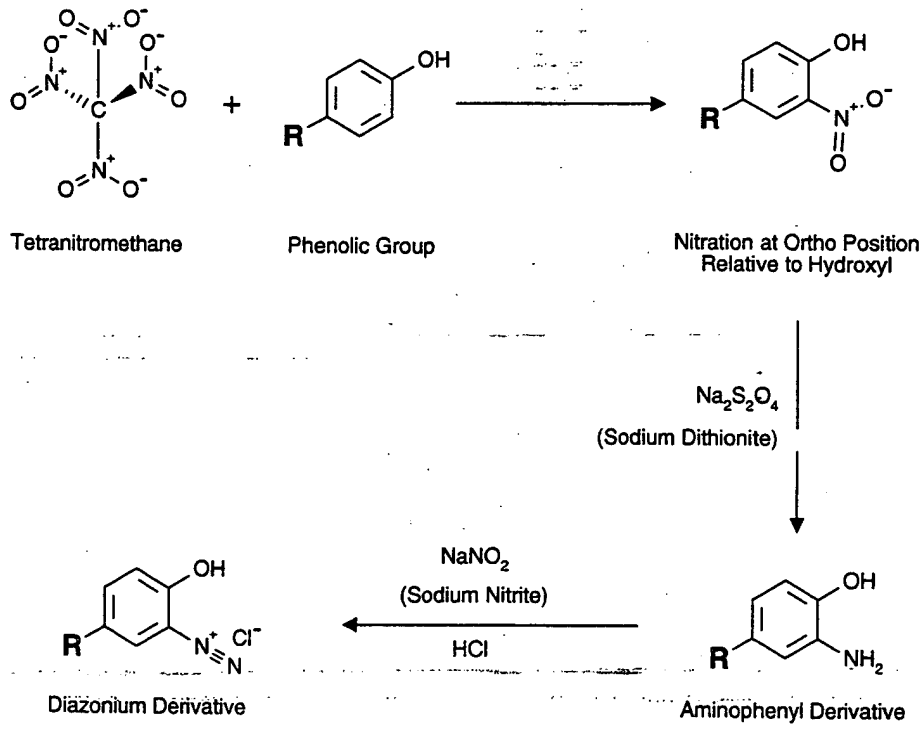


Figure 89 Phenolic compounds, such as the side chain of tyrosine residues, may be modified to contain an amine group by nitration followed by reduction to the aminophenyl derivative.

5. Add sufficient sodium dithionite to bring the final concentration in the reaction medium to 0.1 *M*.
6. React for 1 h at room temperature.
7. Purify the aminophenyl derivative by gel filtration or dialysis

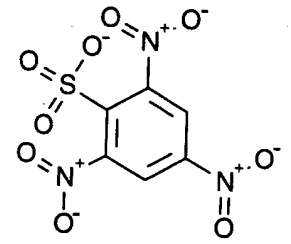
The formation of a diazonium group from the arylamine derivative can be done by treatment with sodium nitrate in HCl (see protocol in Chapter 9, Section 6.1).

Amine Detection Reagents

There are several methods available for the detection or measurement of amine groups in proteins and other molecules. Accurate determination of target amine groups in molecules before or after modification may be important for assessing reaction yield or suitability for subsequent cross-linking procedures. The following methods use commercially available reagents and are easily employed to detect primary amines with simple spectrophotometric measurement.

TNBS

Molecules containing primary amines or hydrazide groups can react with 2,4,6-trinitrobenzenesulfonate (TNBS) to form a highly chromogenic derivative (Fig. 90). This reaction may be used to assay the amine content of compounds by measuring the absorbance of the orange-colored product at 335 nm.



TNBS;
Trinitrobenzene sulfonic acid
MW 293

TNBS has been used to measure the free amino groups in proteins (Habeeb, 1966), as a qualitative check for the presence of amines, sulfhydryls, or hydrazides (Inman and Dintzis, 1969), and to determine specifically the number of ε-amino groups of L-lysine in carrier proteins (Sashidhar *et al.*, 1994).

The following protocol may be used for the measurement of amines in soluble molecules, such as proteins or other macromolecules.

Protocol

1. Dissolve or dialyze the molecule to be assayed into 0.1 *M* sodium bicarbonate, pH 8.5, at a concentration of 20–200 μg/ml (for large molecules like proteins) or 2–20 μg/ml (for small molecules like amino acids).
2. Dissolve TNBS in 0.1 *M* sodium bicarbonate, pH 8.5, at a concentration of 0.01% (w/v). Prepare fresh. Note: TNBS may be prepared as a stock solution in ethanol at a concentration of 1.5%. This solution is stable to long-term storage

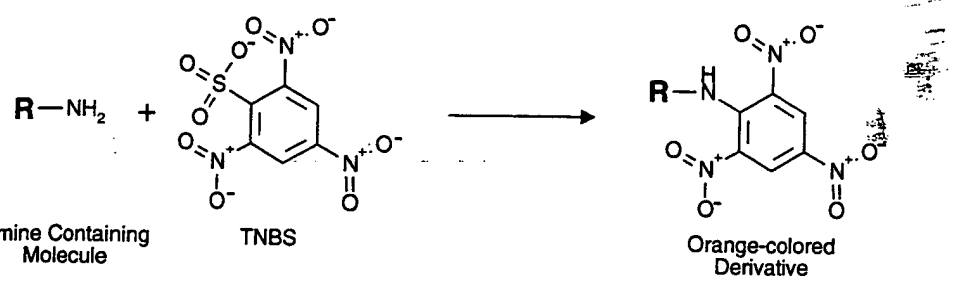


Figure 90 TNBS may be used to detect or quantify amine groups through the production of a chromogenic derivative.

and may be diluted as needed in the bicarbonate buffer to the required concentration.

3. Add 0.5 ml of TNBS solution to 1 ml of each sample solution. Mix well.
4. Incubate at 37°C for 2 h.
5. Add 0.5 ml of 10% SDS and 0.25 ml of 1 *N* HCl to each sample.
6. Measure the absorbance of the solutions at 335 nm. Determination of the number of amines present in a particular sample may be done by comparison to a standard curve generated by use of an amine containing compound (i.e., an amino acid) dissolved at a series of known concentrations in the bicarbonate sample buffer and assayed under identical conditions.

OPA

O-Phthaldialdehyde (OPA) is an amine detection reagent that reacts in the presence of 2-mercaptoethanol to generate a fluorescent product (for preparation, see Section 4.1, 2-mercaptoethanol) (Fig. 91). The resultant fluorophore has an excitation wavelength of 360 nm and an emission point at 455 nm. OPA can be used as a sensitive detection reagent for the HPLC separation of amino acids, peptides, and proteins (Fried *et al.*, 1985). It is also possible to measure the amine content in proteins and other molecules using a test tube or microplate format assay with OPA. Detection limits are typically in the microgram per milliliter range for proteins.

Protocol

1. Prepare a series of standards, preferably consisting of serial dilutions of the substance to be measured, dissolved in water or non-amine-containing buffer.

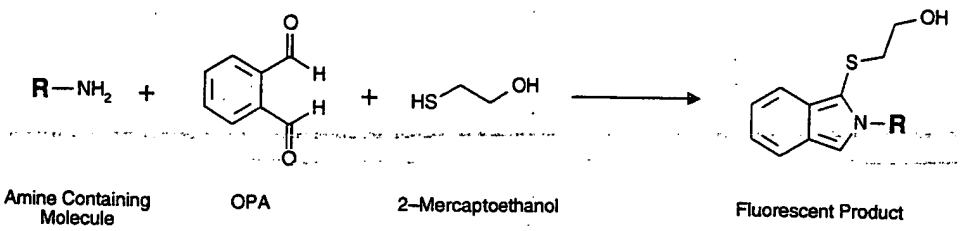


Figure 91 OPA reacts with amines to form a fluorescent product.

The concentration range of the standards can be anywhere between about 500 ng/ml and 1 mg/ml.

2. Prepare the samples dissolved in water or non-amine-containing buffer at an expected concentration level that falls within the standard curve range. The assay can tolerate the presence of most buffer salts, denaturants, and detergents. However, the standard curve should be run in the same buffer environment as the samples to obtain consistent response.
3. To a set of labeled tubes, add 2 ml of OPA reagent (Pierce) and 200 µl of the appropriate standard or sample. Mix well. If using a microplate format, scale back these quantities 10-fold to fit in the microwells.
4. Measure the fluorescence of each sample and standard using an excitation wavelength of 360 nm and an emission wavelength of 436 nm (or using a filter close to the 436 to 455-nm range).
5. Determine the concentration of the samples by comparison to the standard curve. Since the assay measures the presence of amine groups, the results may be correlated to the relative amount of amines available.

4.4. Introduction of Aldehyde Residues

The formation of an aldehyde group on a macromolecule can produce an extremely useful derivative for subsequent modification or conjugation reactions. In their native state, proteins, peptides, nucleic acids, and oligonucleotides contain no naturally occurring aldehyde residues. There are no aldehydes on amino acid side chains, none introduced by post-translational modifications, and no formyl groups on any of the bases or sugars of DNA and RNA. To create reactive aldehydes at specific locations within these molecules opens the possibility of directing modification reactions toward discrete sites within the macromolecule.

There are two basic ways of introducing aldehyde residues in biological macromolecules: (1) oxidation of carbohydrates or adjacent diol containing molecules and (2) modification of available amino groups with reagents that contain or produce aldehydes. In both cases, aldehydes can be created that will allow easy conjugation to amine containing molecules by Schiff base formation and reductive amination (Chapter 2, Section 5.3 and Chapter 3, Section 4). The following sections describe these methods.

Periodate Oxidation of Glycols and Carbohydrates

Carbohydrates and other biological molecules that contain polysaccharides, such as glycoproteins, can be specifically modified at their sugar residues to produce reactive formyl functionalities. With proteins, this method often allows modification to occur only at specific locales, usually away from critical active centers or binding sites.

Periodate oxidation is perhaps the simplest route to transforming the relatively unreactive hydroxyls of sugar residues into amine reactive aldehydes. Periodate cleaves carbon—carbon bonds that possess adjacent hydroxyls, oxidizing the —OH groups to form highly reactive aldehydes (Bobbit, 1956; Rothfus and Smith, 1963). Terminal *cis*-glycols result in the loss of one carbon atom as formaldehyde and the creation of an aldehyde group on the former No. 2 carbon atom. Varying the concentration of

sodium periodate during the oxidation reaction gives some specificity with regard to what sugar residues are modified. Sodium periodate at a concentration of 1 m*M* at 0°C specifically cleaves only at the adjacent hydroxyls between carbon atoms 7, 8, and 9 of sialic acid residues (Van Lenten and Ashwell, 1971; Wilchek and Bayer, 1987). The product is the formation of one aldehyde group on the No. 7 carbon and liberation of two molecules of formaldehyde (Fig. 92).

Since sialic acid is a frequent terminal sugar constituent of the polysaccharide trees on glycoproteins, this method selectively forms reactive aldehydes on the most accessible parts for subsequent modifications. The carbohydrate polymer of a protein provides a long spacer arm that can be used to conjugate another large macromolecule, such as a second protein, with little steric problems.

Oxidation of polysaccharides using 10 m*M* or greater concentrations of sodium periodate results in the cleavage of adjacent hydroxyl-containing carbon—carbon bonds on other sugars besides just sialic acid residues (Lotan *et al.*, 1975). High concentrations of periodate result in sugar ring opening and the creation of many aldehydes on each polysaccharide tree.

Using these methods, carbohydrate-containing proteins may be altered to contain aldehydes for conjugation with other proteins or for detection using hydrazide-containing probes (Chapter 13, Section 5). The aldehydes thus formed then can be coupled to other amine-containing molecules by Schiff base formation and reductive amination (chapter 2, Section 5.3 and Chapter 3, Section 4). For instance, the enzyme horseradish peroxidase (HRP) can be activated with periodate for conjugation with antibodies (Nakane and Kawaoi, 1974). Alternatively, such reactive formyl groups may be conjugated to hydrazide-containing molecules to form hydrazone bonds

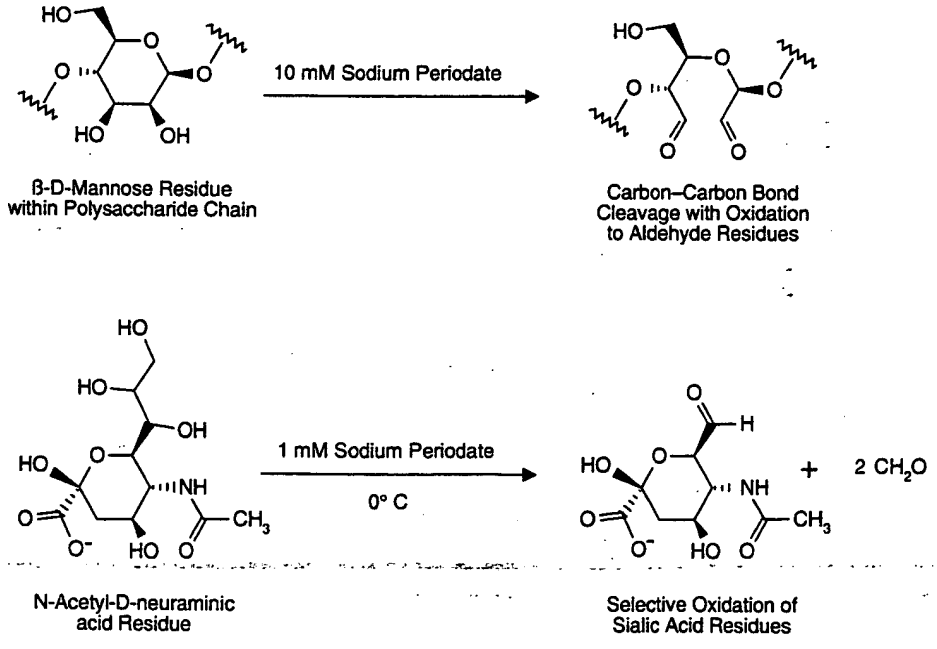


Figure 92 The reaction of sodium periodate with sugar residues can produce aldehydes for conjugation reactions.

(Chapter 4, Section 8, Chapter 10, Section 1.3, and Chapter 17, Section 2.1). Cell surface polysaccharides may be probed with hydrazide-containing reagents for sialic acid groups or total glycoconjugates. Glycoproteins or glycopeptides in solution also may be tagged in this manner. Gangliosides and other glycolipids may be modified with hydrazide reagents as well (Spiegal *et al.*, 1982).

Protocol

1. The glycoprotein or *cis*-diol-containing molecule is dissolved in deionized water or a buffer at physiological pH. Sodium phosphate buffer (0.01–0.1 *M*), pH 7, is an appropriate choice. When oxidizing cell surface glycoconjugates, use a buffer suitable for cellular stability requirements. Avoid amine-containing buffers such as Tris and glycine, because they may interact with the aldehyde groups as they are formed. For glycoproteins in solution, a concentration range of 1–10 mg/ml will produce acceptable results in this procedure. For sialic acid modification, place the sample in ice to cool to near 0°C.
2. Dissolve sodium periodate (MW 213.91) in water at a concentration of 10 mg/ml (0.046 *M*). Protect from light. To obtain approximately a 1 m*M* concentration of sodium periodate in the reaction solution (suitable for oxidizing only sialic acid residues), add 21.8 μl of this stock solution to each milliliter of the glycoprotein solution to be oxidized. Maintain the solution on ice. For general oxidation of carbohydrates other than just sialic acid, add 218 μl of the stock solution to obtain an approximate final concentration of 10 m*M* periodate in the reaction. Use room temperature conditions for general carbohydrate oxidation. Wrap the vial containing the reaction solution with aluminum foil to protect from light. The use of an amber vial is suitable for this purpose.
3. React for 15–30 min at room temperature.
4. Quench the reaction by the addition of 0.1 ml of glycerol per milliliter of reaction solution. Alternatively, the reaction may be stopped by immediate gel filtration on a Sephadex G-25 column. The dextran beads of the chromatography support will react with sodium periodate to quench excess reagent. To quench the reaction with cellular samples, wash the cells with buffer to remove remaining traces of periodate.

Oxidase Modification of Sugar Residues

Another method of forming aldehyde groups on carbohydrates and glycoproteins involves the use of specific sugar oxidases. These enzymes only affect the monosaccharide they are specific toward, leaving other sugar residues within polysaccharides alone. Probably the most often used oxidase for this purpose is galactose oxidase, which can form C-6 aldehydes on terminal D-galactose or *N*-acetyl-D-galactose residues (Avigad *et al.*, 1962) (Fig. 93). When galactose residues are penultimate to sialic acid residues, another enzyme, neuraminidase, must be used to remove the sialic acid sugars and expose galactose as the terminal residue (Wilchek and Bayer, 1987). The specificity of using glycosidases to create aldehyde residues on carbohydrates may be the method's greatest advantage. However, the use of a simple chemical reagent such as sodium periodate still may be the easiest way to create aldehydes on carbohydrates (Section 4.4).

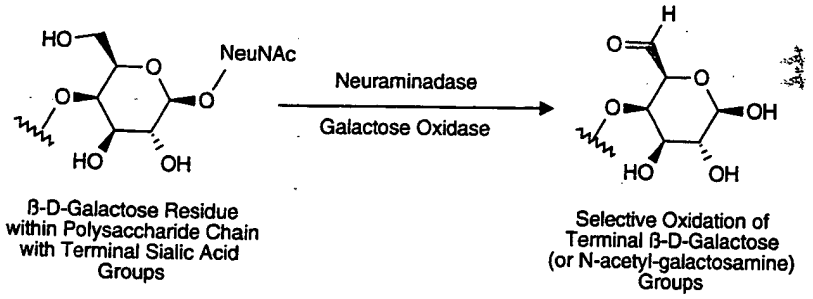


Figure 93 Galactose oxidase may be used to transform specifically the C-6 hydroxyl group of galactose residues into an aldehyde.

The following protocol was used by Wilchek and Bayer (1987) to label cell surface galactose residues.

Protocol

1. Prepare a 5% cell suspension in an appropriate buffer. Avoid amine-containing buffers as these will interact with aldehydes.
2. Add 0.05 units of *Vibrio cholerae* neuraminidase and 5 units of galactose oxidase per milliliter of cell suspension.
3. Incubate for 60 min at 37°C.

Modification of Amines with NHS-Aldehydes (SFB and SFPA)

Succinimidyl *p*-formylbenzoate (SFB) and succinimidyl *p*-formylphenoxyacetate (SFPA) are amine-reactive reagents that contain terminal aldehyde residues. Their NHS ester ends react with primary amines in proteins and other molecules at pH 7–9 to yield amide linkages (Chapter 2, Section 1.4) (Fig. 94.) The resulting formyl derivatives may be utilized to couple to other amine or hydrazide-containing molecules (Galardy *et al.*, 1978; Kraehenbuhl *et al.*, 1974). In particular, SFB can be used to produce aldehyde groups on alkaline phosphatase for conjugation with 5′-hydrazide-modified DNA for use in hybridization assays (Chapter 17, Section 2.4) (Ghosh *et al.*, 1989). SFB and SFPA are insoluble in water, but may be predissolved in DMF or acetonitrile before adding a small quantity to an aqueous reaction mixture. Both reagents contain aromatic phenyl rings and have absorptivity at wavelengths less than 300 nm. Their structures may contribute a significant degree of hydrophobicity to

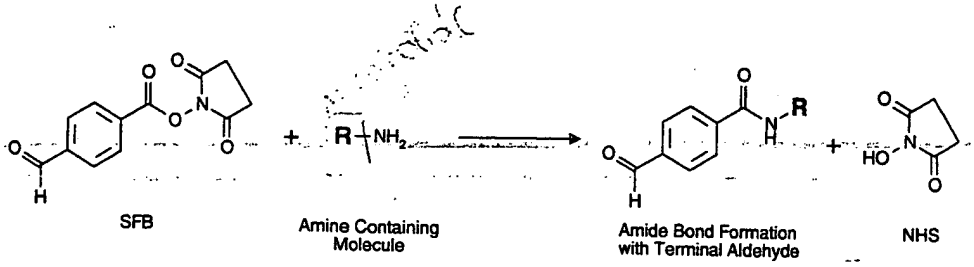
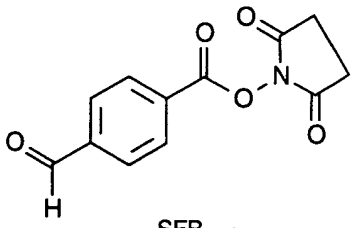


Figure 94 SFB reacts with primary amines to form amide bond derivatives containing aldehyde groups.

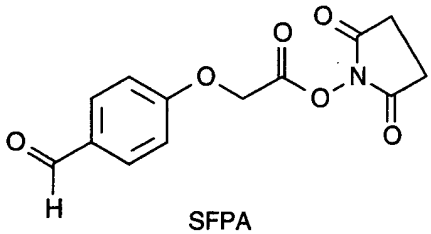
macromolecules being modified, especially if high-density couplings are achieved. For this reason, modified proteins and other soluble molecules may have a tendency to precipitate if modification is done too heavily. The optimal amount of modification may have to be adjusted to maintain solubility in each application.

Protocol

1. Dissolve a macromolecule containing amine groups at a concentration of 1–10 mg/ml in a buffer having a pH of 7–9 (i.e., 0.1 *M* sodium phosphate, pH 7.5). Avoid amine-containing or nucleophilic buffers such as Tris, glycine, or imidazole (see Chapter 2, Section 1.4).



SFB
Succinimidyl-p-formyl benzoate
MW 247



SFPA
Succinimidyl-p-formylphenoxyacetate
MW 277

2. Dissolve SFB or SFPA (Molecular Probes) in DMF. The concentration should be such that a small aliquot can be added to the reaction medium to obtain about a 10-fold excess of modifying reagent over the amount of amines to be modified. Add no more than 100 μl of the modifier/DMF solution to each milliliter of the macromolecule solution prepared in (1).
3. React for 2 h at room temperature.
4. Purify the modified macromolecule from excess reagent and reaction by-products by dialysis or gel filtration.

Modification of Amines with Glutaraldehyde

Amino groups on proteins may be reacted with the bis-aldehyde compound glutaraldehyde to form activated derivatives able to cross-link with other proteins. The reaction mechanism for this modification proceeds by one of several possible routes. In the first option, one of the aldehyde ends can form a Schiff base linkage with ε-amines

or α-amines on proteins to leave the other aldehyde terminal free to conjugate with another molecule. Alternatively, a glutaraldehyde polymer may undergo vinyl addition to create stable secondary amine bonds, leaving the aldehydes exposed for subsequent reductive amination reactions. Finally, a cyclized form of glutaraldehyde also may react with the ε-amines of two neighboring lysine side chains to form a quaternary pyridinium cross-link (Fig. 95).

Schiff base interactions between aldehydes and amines typically are not stable enough to form irreversible linkages. These bonds may be reduced with sodium cyanoborohydride or a number of other suitable reductants (Chapter 3, Section 4) to form permanent secondary amine bonds. However, proteins cross-linked by glutaraldehyde without reduction nevertheless show stabilities unexplainable by simple Schiff base

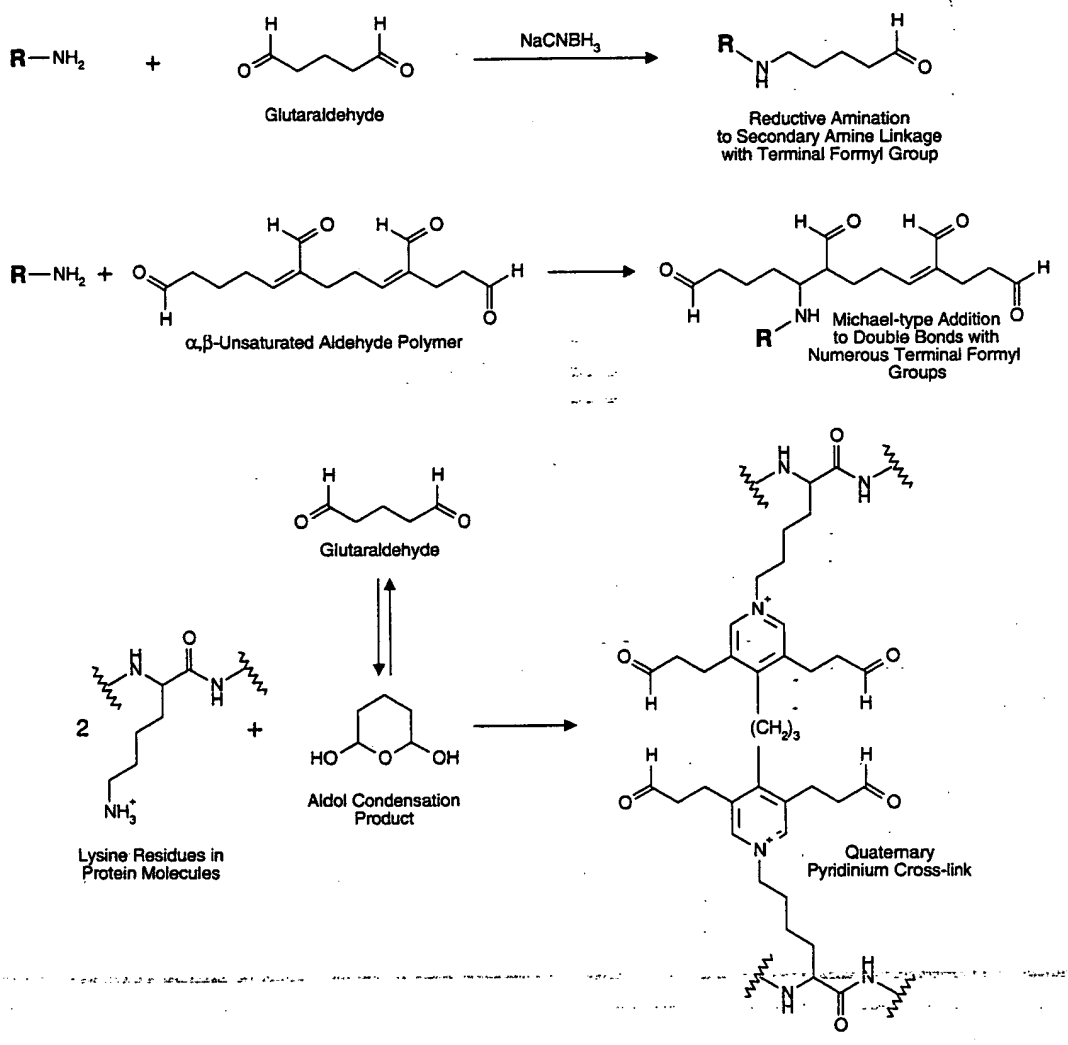


Figure 95 Glutaraldehyde can undergo complex reactions with amine groups, resulting in aldehyde-containing derivatives that can be used in conjugation reactions.

formation. The stability of such unreduced glutaraldehyde conjugates has been postulated to be due to the vinyl addition mechanism, which does not depend on the creation of Schiff bases.

Glutaraldehyde modification readily proceeds at alkaline pH. The higher the pH, the more efficient is Schiff base formation. Using a reductant like sodium cyanoborohydride that does not affect the aldehyde groups, while efficiently transforming the Schiff base into a secondary amine, provides the best possible yields. In many cases, the degree of glutaraldehyde-induced cross-links is so severe that conjugate precipitation occurs. This is especially well documented in antibody–enzyme conjugation schemes employing this reagent (Chapter 10, Section 1.2).

Glutaraldehyde also can be used to create aldehydes on amine-containing polymers. The use of this reagent in derivatizing chromatography supports and other soluble polymers is well known (Hermanson *et al.*, 1992).

The following protocol may be used as the first stage of a two-step glutaraldehyde conjugation reaction. In this initial reaction, glutaraldehyde modification converts available protein amines into reactive formyl groups. The subsequent addition of a second protein or another amine-containing molecule causes this activated protein to cross-link with the amines and form a conjugate. Glutaraldehyde also may be used in single-step conjugation procedures where the aldehyde-modified protein is not isolated before addition of a second protein. In single-step conjugations both proteins to be cross-linked are together in solution and glutaraldehyde is added to effect cross-linking (Chapter 10, Section 1.2).

Protocol

1. Dissolve the protein or other amine-containing macromolecule to be modified at a concentration of 1–10 mg/ml in a buffer having a pH from 7 to 10. The higher the pH, the more efficiently Schiff base formation will occur. Phosphate, borate, and carbonate buffers at 0.01–0.1 *M* are acceptable. Avoid amine-containing buffers like Tris and glycine, since they will react with glutaraldehyde.
2. Add a quantity of glutaraldehyde equal to a 10-fold molar excess over the amount of amines to be modified. A typical concentration of glutaraldehyde in the reaction mixture is 1.25%. In some cases, trial experiments will have to be done to check for solubility of the resultant modified protein. Scale back the quantity of glutaraldehyde added if precipitation occurs.
3. React for at least 2 h at 4°C.
4. Quickly isolate the modified protein by gel filtration using Sephadex G-25 or the equivalent.

In some cases, the modified protein may be stored for long periods before conjugation with another amine-containing molecule by immediate freezing and lyophilization. If stability is a problem, however, the modified protein should be conjugated immediately.

4.5. Introduction of Hydrazide Functional Groups

Hydrazide-containing reagents can be used for probing or conjugation of carbonyl-containing compounds, including macromolecules possessing aldehydes and ketones.

Fluorescent or enzymatic probes containing hydrazide functional groups can be used to assay or label carbohydrates, glycoproteins, the polysaccharide portion of cell surfaces, gangliosides, and glycoconjugates on blots (Wilchek and Bayer, 1987; Lotan *et al.*, 1975; Spiegal *et al.*, 1982; Hurwitz *et al.*, 1980; Gershoni *et al.*, 1985). Multivalent forms of hydrazide reagents created by modifying enzymes, ferritin, and polymers such as dextran and polypeptides with *bis*-hydrazides can be used to target formyl groups with high avidity and sensitivity (Roffman *et al.*, 1980; Kaplan *et al.*, 1983).

The creation of hydrazide probes most often is based on the derivatization of a detectable molecule with a *bis*-hydrazide compound. Although hydrazine itself (in the form of hydrazine hydrate) can be used in a methanolic solution to modify activated carboxylate molecules forming hydrazides, the availability of the bifunctional hydrazides provides a built-in spacer to accommodate greater steric accessibility.

The following protocols make use of the compounds adipic acid dihydrazide and carbohydrazide to derivatize molecules containing aldehydes, carboxylates, and alkylphosphates. The protocols are applicable for the modification of proteins, including enzymes, soluble polymers such as dextrans and poly-amino acids, and insoluble polymers used as microcarriers or chromatographic supports.

The addition of hydrazide groups into macromolecules containing aldehydes, carboxylates, or alkylphosphates has the effect of increasing the pI or net charge. In the case of carboxylates or alkylphosphates, blocking these groups with hydrazide compounds eliminates the negative charge contribution of the original functional group and adds a potential positive charge contribution due to the terminal hydrazide. The consequence of raising the pI of a macromolecule can have dramatic effects on the molecule's conformation and activity or on its relative nonspecificity in assay systems due to the presence of additional positive charge. For instance, the modification of avidin with adipic acid dihydrazide by coupling through the protein's carboxylate groups significantly increases the net charge of an already highly cationic molecule, and therefore increases its overall cross-reactivity in avidin–biotin assays (Chapter 13, Section 5).

Modification of Aldehydes with Bis-hydrazide Compounds

Aldehyde-containing macromolecules will react spontaneously with hydrazide compounds to form hydrazone linkages. The hydrazone bond is a form of Schiff base that is more stable than the Schiff base formed from the interaction of an aldehyde and an amine. The hydrazone, however, may be reduced and further stabilized by the same reductants utilized for reductive amination purposes (Chapter 3, Section 4). The addition of sodium cyanoborohydride to a hydrazide–aldehyde reaction drives the equilibrium toward formation of a stable covalent complex. Mallia (1992) has found that adipic acid dihydrazide derivatization of periodate-oxidized dextran (containing multiple formyl functionalities) proceeds with much greater yield when sodium cyanoborohydride is present.

The reaction of an excess of adipic acid dihydrazide with aldehyde groups present on proteins or other molecules will result in modified proteins containing alkylhydrazide groups (Fig. 96). Another bis-hydrazide compound, carbohydrazide, also may be employed with similar results, except that the spacer afforded through its use is considerably shorter. Target aldehydes may be created on macromolecules according

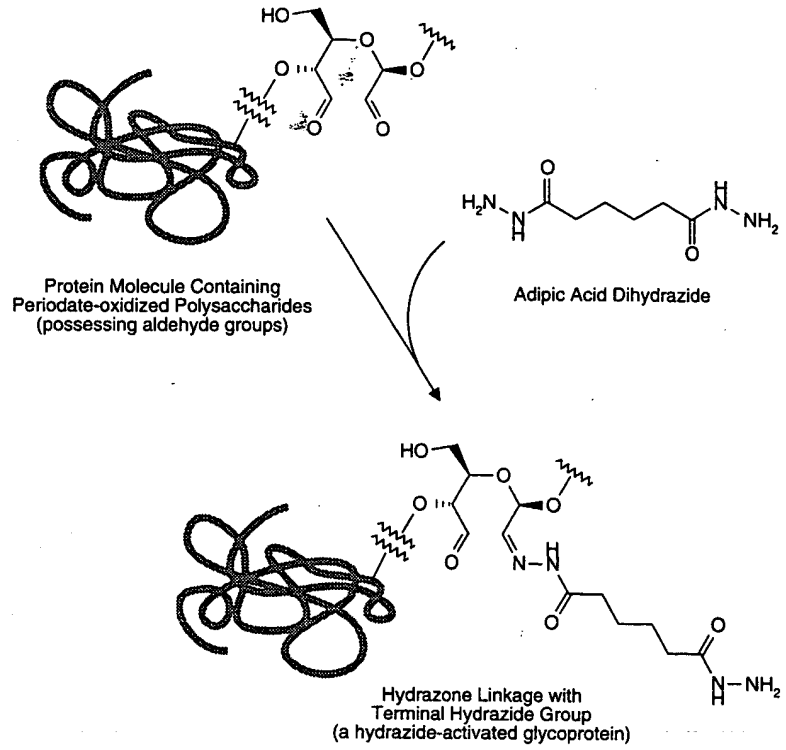


Figure 96 Glycoproteins that have been treated with sodium periodate to produce aldehyde groups can be further modified with adipic acid dihydrazide to result in a hydrazide derivative.

to the protocols described in Section 4.4. Thus, glycoproteins and other molecules containing polysaccharide may be periodate-oxidized to contain formyl groups and then modified with a *bis*-hydrazide compound to create the hydrazide-activated reagent. Modification of proteins through carbohydrate residues obviates the blocking of negatively charged carboxylates and only adds limited numbers of hydrazides at discrete portions of a molecule. The enzyme horseradish peroxidase is conveniently modified with hydrazide functional groups using this approach (Chapter 16, Section 2.4).

Protocol

1. Dissolve a macromolecule (such as a protein) containing aldehyde functional groups in a buffered solution at a pH of about 7–8.5 and at a concentration of about 1–10 mg/ml. To modify a molecule to contain aldehyde groups, see Section 4.4. Phosphate, carbonate, borate, or similar buffers adjusted to this pH range work well. Avoid amine-containing buffers (i.e., glycine or Tris) or other components containing strong nucleophiles, since these may react with the aldehydes. Higher pH environments enhance the formation of hydrazone bonds and generally increase the yield of complex.
2. Add a quantity of adipic acid dihydrazide or carbohydrazide (Aldrich) to the

protein solution to obtain at least a 10-fold molar excess over the amount of aldehyde functional group present. If the concentration of aldehydes is unknown, the addition of 32 mg adipic acid dihydrazide per milliliter of the protein solution to be modified should work well.

3. React for 2 h at room temperature. Although hydrazone formation does not require the addition of a reductant to create a linkage, including sodium cyanoborohydride in the reaction considerably increases the yield and stability of bonds formed. If the presence of a reducing agent will not cause harm to the macromolecule being modified, the addition of 10 µl of 5 *M* sodium cyanoborohydride (Sigma) per milliliter of reaction solution may be done. Caution: cyanoborohydride is extremely toxic. All operations should be done with care in a fume hood. Also, avoid any contact with the reagent, as the 5 *M* solution is prepared in 1 *N* NaOH.
4. Purify the modified protein by dialysis or gel filtration.

Hydrazide-activated proteins are stable to long-term storage at 4°C in the presence of a preservative (0.05% sodium azide) or in a frozen or lyophilized state.

Modification of Carboxylates with Bis-hydrazide Compounds

Carboxylic acids may be covalently modified with adipic acid dihydrazide or carbohydrazide to yield stable imide bonds with extending terminal hydrazide groups. Hydrazide functionalities do not spontaneously react with carboxylate groups the way they do with formyl groups (Section 4.5). In this case, the carboxylic acid first must be activated with another compound that makes it reactive toward nucleophiles. In organic solutions, this may be accomplished by using a water-insoluble carbodiimide (Chapter 3, Section 1.4) or by creating an intermediate active ester, such as an NHS ester (Chapter 2, Section 1.4).

In aqueous solutions, the easiest method for forming this type of bond is by use of the water-soluble carbodiimide EDC (Chapter 3, Section 1.1). For proteins and other water-soluble macromolecules, EDC reacts with their available carboxylate groups to form an intermediate, highly reactive, O-acylisourea. This active ester species may further react with nucleophiles such as a hydrazide to yield a stable imide product (Fig. 97).

Most proteins contain an abundance of carboxylic acid groups from C-terminal functional groups and aspartic and glutamic acid side chains. these groups are readily modified with *bis*-hydrazide compounds to yield useful hydrazide-activated derivatives. Both carbohydrazide and adipic acid dihydrazide have been employed in forming these modifications using the carbodiimide reaction (Wilchek and Bayer, 1987).

Protocol

1. Dissolve 32 mg of adipic acid dihydrazide per milliliter of 0.1 *M* sodium phosphate, 0.15 *M* NaCl, pH 7.2.
2. Dissolve 5 mg of the protein or other macromolecule to be modified per milliliter of the above solution.
3. Add 16 mg EDC and react at room temperature for 4 h.
4. Purify the modified protein by dialysis or gel filtration.

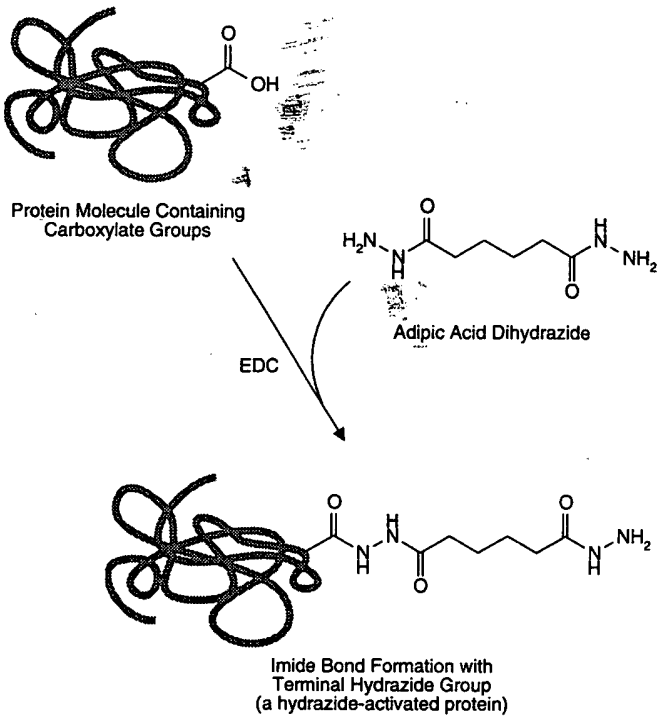


Figure 97 Carboxylate groups on proteins may be modified with adipic acid dihydrazide in the presence of a carbodiimide to produce hydrazide derivatives.

Modification of Alkylphosphates with Bis-hydrazide Compounds

Alkylphosphate groups such as those present at the 5′ end of RNA and DNA molecules may be specifically modified with bis-hydrazide compounds. Mediated by the addition of the water-soluble carbodiimide EDC and imidazole, adipic acid dihydrazide or carbohydrazide will react with the phosphate group in a two-step process to form phosphoramidate bonds with short linker arms containing terminal hydrazides (Fig. 98) (Ghosh *et al.*, 1989). In the first stage, EDC activates the phosphate group forming a short-lived, but highly reactive, phosphodiester species, which in turn reacts with a molecule of imidazole to form a longer-lived, active phosphorimidazolide. The

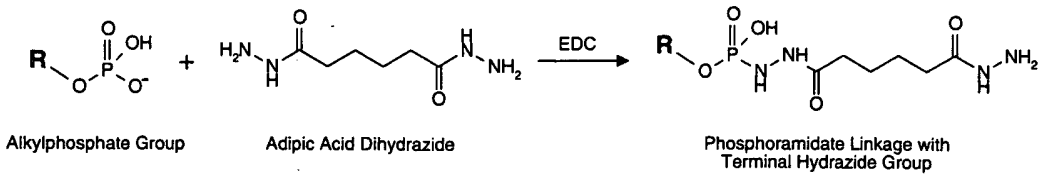


Figure 98 Phosphate groups may be modified with adipic acid dihydrazide in the presence of a carbodiimide to produce hydrazide derivatives. This is a common modification route for the 5′-phosphate group of oligonucleotides.

second stage involves addition and attack of the hydrazide nucelophile, releasing imidazole and forming the phosphoramidate bond. In a modification of the two-stage reaction, Zanocco *et al.* (1993) developed a single-pot reaction in which the alkylphosphate molecule is reacted in the presence of EDC, imidazole, and the bis-hydrazide compound. The modification reaction proceeds rapidly at room temperature.

Protocol

1. Weigh out 1.25 mg of the carbodiimide EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; Pierce) into a microfuge tube.
2. Add to the tube 7.5 µl of RNA or DNA containing a 5′ phosphate group. The concentration of the oligonucleotide should be 7.5–15 nmol or total of about 57–115.5 µg. Also, immediately add 5 µl of 0.25 *M* adipic acid dihydrazide or carbohydrazide dissolved in 0.1 *M* imidazole, pH 6. Because EDC is labile in aqueous solutions, the addition of the oligo and bis-hydrazide/imidazole solutions should occur quickly.
3. Mix by vortexing, then place the tube in a microcentrifuge and spin for 5 min at maximal rpm.
4. Add an additional 20 ml of 0.1 *M* imidazole, pH 6. Mix and react for at least 2 h at room temperature. The additional buffer prevents pH drift during the carbodiimide reaction.
5. Purify the hydrazide-labeled oligo by gel filtration on Sephadex G-25 using 10 m*M* sodium phosphate, 0.15 *M* NaCl, 10 m*M* EDTA, pH 7.2. The hydrazide-containing probe now may be used to conjugate with a molecule containing an aldehyde reactive group.

5. Blocking Specific Functional Groups

It is often necessary to block specific groups on macromolecules to prevent them from participating in modification or conjugation reactions. In most blocking procedures, a chemical group is covalently coupled to an undesired functional group on the macromolecule to mask or eliminate its reactivity. In this sense, the modification is done with a compound that is relatively inert in whatever application for which the macromolecule is intended. The blocking agent is usually a small organic compound containing a functional group able to couple with the group to be masked. The blocking molecule may contain another functional group of its own, converting the blocked group into a chemical function of another type, but this conversion is all right, providing the newly created function does not interfere in subsequent reactions or applications.

In some cases, a blocking procedure is done to direct a conjugation reaction to discrete sites in a macromolecule. In other instances, blocking a group on one of two macromolecules can prevent self-polymerization and promote the desired intermolecular conjugation. For instance, HRP can be blocked with an amine-specific coupling reagent prior to periodate oxidation to prevent the reactivity of its two amino groups during subsequent conjugation with an antibody molecule (Chapter 10, Section 1.3).

In other uses of blocking reagents, proteins dissociated into subunits by the use of denaturants and disulfide reductants may be prevented from reassociation or oxidation of their sulfhydryls by blocking the —SH groups with the appropriate reagent. Alternatively, sulfhydryls may be blocked on a protein prior to activation with a heterobifunctional cross-linking agent that contains amine-reactive and sulfhydryl-reactive ends. The amine-reactive end will couple to the amines of the protein without reaction of the sulfhydryl-reactive end. This can prevent oligomer formation during the activation process and thus ensure that the sulfhydryl-reactive function is available for conjugation with the desired molecule.

Controlled functional studies of a protein's active center also may be done by blocking specific groups and observing its effect on activity. Often, this blocking procedure is performed through the use of a reversible blocking agent subsequently to regenerate activity, therefore demonstrating that the effect was directed at functional groups present in the active site (Perham and Jones, 1967).

Blocking also may be done to quench further modification or conjugation through a targeted group. In addition, after a conjugation reaction, excess functional groups may be masked from nonspecifically reacting with other molecules. For instance, periodate-oxidized glycoproteins may still contain aldehyde groups after conjugation with another protein by reductive amination. Blocking the aldehydes with a small amine-containing molecule prevents unwanted reactions from occurring when the conjugate is used in an assay or targeting operation. This is also true of excess sulfhydryl groups, which may undergo disulfide interchange with other sulfhydryl molecules subsequent to a conjugation reaction. Blocking these groups with the appropriate reagent prevents this type of side reaction from occurring.

Blocking of amine groups on proteins also has been used to create a sensitive reagent for measuring protease activity (Hatakeyama *et al.*, 1992). With nearly all the primary amines of casein blocked, an amine detection reagent such as trinitrobenzene sulfonic acid (TNBS) will only minimally react with the protein and form its typical orange derivative. As proteases cleave the protein, however, primary α-amines are created from cleavage of the α-chain peptide bonds, and TNBS can react with them. The more protease activity present, the more color is formed.

The choice and application of a specific blocking reagent can produce a modified macromolecule with unique and useful properties. Many of the common blocking reagents are discussed in this section. Beyond the scope of this book, however, is a discussion of the numerous blocking agents used in peptide or nucleic acid synthesis to block temporarily specific reactive groups during growth of the polymer chain.

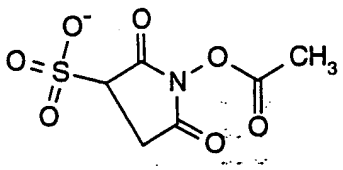
5.1. Blocking Amine Groups

The amine functional groups most commonly found in macromolecules are primary amines such as those at the N-terminal of polypeptide chains (α-amines) and the side-chain ε-amino groups of lysine residues. Several acylation reagents can effectively block these primary amines, some of which are reversible under the right conditions. It should be noted that the cyclic anhydrides mentioned in this section react with amino groups to form amide bonds, opening the anhydride ring and effectively transforming the amine function into a carboxylate. There are additional cyclic anhydrides described in Section 4.2 that also create carboxylates from amines, but in this section the

two discussed, maleic anhydride and citraconic anhydride, both are reversible and designed more for temporary masking than permanent blocking. For more stable blocking of amines, sulfo-NHS acetate and acetic anhydride are the best choices.

Sulfo-NHS Acetate

Sulfo-NHS acetate is the *N*-hydroxysulfosuccinimide ester of acetic acid. The NHS ester end provides high reactivity with the amino groups of proteins at a pH range of 7–9, acylating the amines and forming nonreversible acetamide modifications (Fig. 99). The sulfonate derivative of the NHS ester provides good water solubility to the reagent. Thus, the compound can be added directly to an aqueous solution of the protein to be blocked, or a stock solution may be prepared and a small aliquot added to the reaction medium. Stock solutions should be dissolved rapidly and use immediately. In aqueous solutions, the main competing reaction is hydrolysis of the active ester to release nonreactive sulfo-NHS and acetic acid. The use of a 10- to 50-fold molar excess of sulfo-NHS acetate over the molar amount of groups to be blocked should provide good yields of acylated amines. Reaction buffers should contain no extraneous amines that could cross-react with the sulfo-NHS acetate. Avoid Tris-, glycine-, and imidazole-containing buffers. Phosphate, borate, or bicarbonate buffers work well at a concentration of 0.05–0.1 *M*. React for at least 1 h at room temperature.



Sulfo–NHS-Acetate
MW 259.17

Protocol

1. Dissolve the protein or other amine-containing macromolecule at a concentration of 1–10 mg/ml in 0.1 *M* sodium phosphate, 0.15 *M* NaCl, pH 7.5.
2. Add a 25 molar excess of sulfo-NHS acetate over the amount of amines present in the sample. If the precise amount of amines is not known, adding an equal mass of reagent to the mass of protein will provide a large excess of reactivity to completely block all amines.
3. React at room temperature for at least 1 h.
4. Purify the modified protein by dialysis or gel filtration.

Acetic Anhydride

Acetic anhydride is the only monocarboxylic acid anhydride that is important in modification reactions. The acetylation of the amino groups of proteins can be made relatively specific if the reaction is done in saturated sodium acetate, since the O-acetyltyrosine derivative is unstable to an excess of acetate ions (Fraenkel-Conrat, 1959). The tyrosine derivative rapidly hydrolyzes in alkaline reaction conditions, even

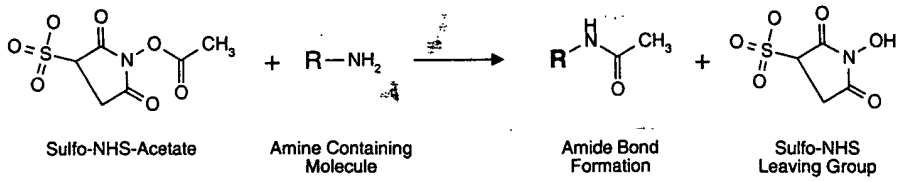
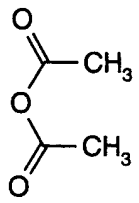


Figure 99 Sulfo-NHS acetate may be used to block amine groups, forming permanent amide bond derivatives.

in the absence of added acetate buffer (Uraki *et al.*, 1957; Smyth, 1967). Treatment with hydroxylamine also cleaves any O-acetyltyrosine modifications, forming acetylhydroxamate, which can be followed by its purple complex with Fe^{3+} at 540 nm (Balls and Wood, 1956).

At physiological pH values, acetylation of amine groups proceeds rapidly, requiring less than an hour to go to completion (Fig. 100).



Acetic Anhydride
MW 102

Protocol

1. Dissolve the macromolecule to be modified at a concentration of 1–10 mg/ml in a buffered solution having a pH between 6.5 and 7.5. Avoid amine-containing buffers such as glycine and Tris. Sodium phosphate buffer at a concentration of 0.1 *M* works well. The addition of an equal volume of a saturated solution of sodium acetate may be done to prevent tyrosine derivatization.
2. Cool the solution on ice. With stirring, add an amount of acetic anhydride equal to the mass of macromolecule to be modified. Alternatively, add a 10-fold molar excess of acetic anhydride over the amount of amines present. The addition of the anhydride slowly or in several aliquots over the course of 1 h will ensure good yield of acetylation.
3. React with stirring for at least 1 h while cooling in an ice bath.
4. Purify the acetylated macromolecule by gel filtration or dialysis.

Citraconic Anhydride

Citraconic anhydride (or 2-methylmaleic anhydride) is a derivative of maleic anhydride that is reversible after acylation of amine groups. At alkaline pH values (pH 7–8) the reagent reacts with amines to form amide linkages with an extending terminal carboxylate. However, at acid pH (3–4), these bonds rapidly hydrolyze to release citraconic acid and free the amine (Dixon and Perham, 1968; Klapper and Klotz,

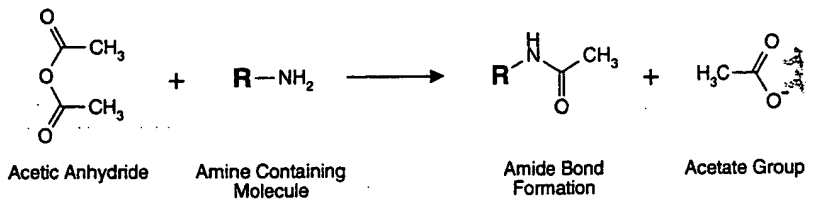


Figure 100 Acetic anhydride reacts with amines to form amide bond derivatives.

1972; Habeeb and Atassi, 1970; Shetty and Kinsella, 1980). Thus, citraconic anhydride is useful in temporarily blocking amine groups while other parts of a molecule are undergoing derivatization. Once the modification is complete, the amines can be then unblocked to create the original structure. See Section 4.2 for additional information and a protocol for modification of proteins with citraconic anhydride.

Maleic Anhydride

Maleic acid is a linear four-carbon molecule with carboxylate groups on both ends and a double bond between the central carbon atoms. The anhydride of maleic acid is a cyclic molecule containing five atoms. Although the reactivity of maleic anhydride is similar to that of other cyclic anhydrides, the products of maleylation are much more unstable toward hydrolysis, and the site of unsaturation lends itself to additional side reactions. Acylation products of amino groups with maleic anhydride are stable at neutral pH and above, but they readily hydrolyze at acid pH values around 3.5 (Butler *et al.*, 1967). Maleylation of sulfhydryls and the phenolate of tyrosine are even more sensitive to hydrolysis. Thus, maleic anhydride is an excellent reversible blocker of amino groups to mask them temporarily from reactivity while another reaction is being done. For additional information and a protocol for the modification of proteins with this reagent, see Section 4.2.

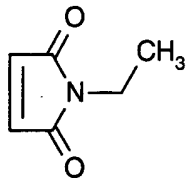
5.2 Blocking Sulfhydryl Groups

The sulfhydryl group is among the most highly reactive of nucleophiles found in biological macromolecules. Cysteine sulfhydryls in proteins undergo covalent reactions rapidly with most of the reactive groups utilized in modification and conjugation reagents. To prevent modification from occurring at these sites, it is often necessary to use a blocking agent that ties up the sulfhydryl and renders it inert toward further reactions.

There are two types of sulfhydryl blocking agents: permanent and reversible. The permanent ones form thioether linkages that do not readily break down. The reversible ones form disulfide bonds that are susceptible to cleavage by the addition of the appropriate reducing agent. Reversible sulfhydryl blockers can be used to mask an —SH group temporarily from modification while a reaction is done at another site. This is especially useful when the sulfhydryl forms a critical part of the active center of a protein. After the final modification is complete, the blocking agent can be removed to regenerate activity.

N-Ethylmaleimide

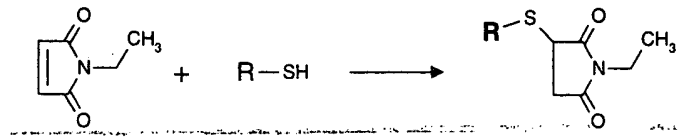
N-Ethylmaleimide (NEM) is an alkylating reagent that reacts with sulfhydryls to form stable thioether bonds (Smyth *et al.*, 1960). Maleimide reactions are specific for sulfhydryl groups in the pH range 6.5–7.5 (Heitz *et al.*, 1968; Smyth *et al.*, 1964; Gorin *et al.*, 1966; Partis *et al.*, 1983) (see Chapter 2, Section 2.2). At higher pH values some cross-reactivity with amino groups takes place (Brewer and Riehm, 1967). One of the carbons adjacent to the double bond undergoes nucleophilic attack by the thiolate anion to generate the addition product (Fig. 101). When sufficient quantities of —SH groups are being blocked, the reaction may be followed spectrophotometrically by the decrease in absorbance at 300 nm as the double bond reacts and disappears. The result is a stable, inert derivative that terminates in the ethyl group. NEM is useful for permanently blocking sulfhydryl residues in proteins and other macromolecules. It has been used for blocking sulfhydryl-containing reagents that interfere in a glucose oxidase assay system (Haugaard *et al.*, 1981).



N-Ethylmaleimide
MW 125.12

Protocol

1. Dissolve the macromolecule containing sulfhydryl groups to be blocked in a buffer having a pH of 6.5–7.5. Sodium phosphate (0.01–0.1 *M*) at pH 7.2 works well. Avoid amine-containing buffers, since an excess of amines may cause some reactivity with the maleimide groups. Also, avoid the presence of sulfhydryl-containing disulfide reductants such as DTT or 2-mercaptoethanol, which will rapidly react with NEM.
2. Add at least a 10-fold molar excess of NEM over the amount of sulfhydryls present in the reaction. Alternatively, add an equal mass of NEM to the amount of macromolecule present. To facilitate the addition of a small quantity of reagent, a more concentrated stock solution may be prepared in buffer and an aliquot added to the reaction medium. Make the stock solution up fresh, and use it immediately to prevent loss of activity due to maleimide group breakdown.



N-Ethylmaleimide | Sulfhydryl Containing Molecule | Thioether Bond Formation

Figure 101 The reaction of *N*-ethylmaleimide with sulfhydryl groups yields a thioether derivative, permanently blocking the thiol.

3. React for 2 h at room temperature.
4. Purify the modified protein by gel filtration or dialysis.

Iodoacetate Derivatives

Iodoacetate (and bromoacetate) can react with several nucleophilic functional groups within proteins. Their relative reactivity toward protein functional groups is sulfhydryl > imidazolyl > thioether > amine. Among α-haloacetate derivatives the relative reactivity is I > Br > Cl > F, with fluorine being almost unreactive. The α-haloacetamides have the same trend of relative reactivities, but will obviously not create a carboxylate functional group. The acetamide derivatives typically are used only as blocking reagents. The bond formed from the reaction of iodoacetamide and a sulfhydryl group is a stable thioether linkage that is not reversible under normal conditions.

Thus, iodoacetamide has the highest reactivity toward cysteine sulfhydryl residues and may be directed specifically for —SH blocking. If iodoacetamide is present in limiting quantities (relative to the number of sulfhydryl groups present) and at slightly alkaline pH, cysteine modification will be the exclusive reaction. For additional information on α-haloacetate reactivities and a protocol for blocking, see Section 4.2.

Sodium Tetrathionate

Sodium tetrathionate ($Na_2S_4O_6$) is a redox compound that under the right conditions can facilitate the formation of disulfide bonds from free sulfhydryls. The tetrathionate anion reacts with a sulfhydryl to create a somewhat stable active intermediate, a sulfenylthiosulfate (Fig. 102). Upon attack of the nucleophilic thiolate anion on this activated species, the thiosulfate (S_2O_3 =) leaving group is removed and a disulfide linkage forms (Pihl and Lange, 1962). The reduction of tetrathionate to thiosulfate *in vivo* was a subject of early study (Theis and Freeland, 1940; Chen *et al.*, 1934).

$Na_2S_4O_6$

Sodium Tetrathionate
MW 270.22

Depending on the proximity of cysteine sulfhydryl groups in proteins, intra- and interchain disulfide formation is possible on reaction with tetrathionate. When neighboring sulfhydryl groups are not close enough to create disulfide linkages, the sulfenylthiosulfate modification is sufficiently stable to block exposed —SH groups temporarily. For sulfhydryls present in the active centers of enzymes, tetrathionate may lead to reversible inactivation (Parker and Allison, 1969). Thus, the reagent may be used to protect certain sulfhydryl residues during modification reactions performed elsewhere on a protein. Using this approach, the enzyme ficin may be temporarily protected with tetrathionate during modification, conjugation, or immobilization reactions done through its amine groups (Liener and Friedenson, 1970). Subsequent treatment with thiol-containing disulfide reducing agents frees the sulfenylthiosulfate and regenerates the sulfhydryl with enzymatic activity. The following protocol is an adaptation of that of Englund *et al.* (1968), used in the purification of ficin.

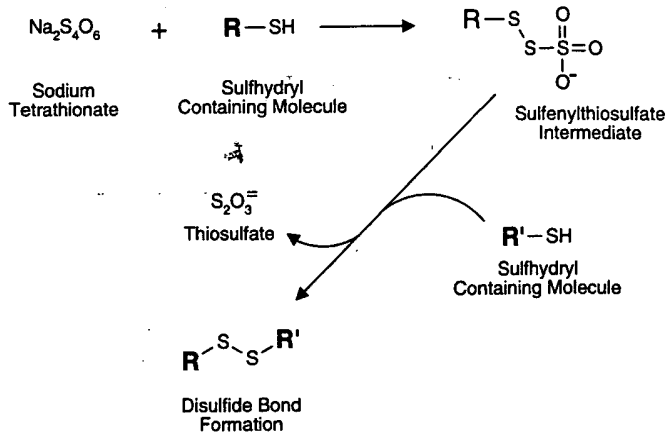


Figure 102 Sodium tetrathionate reacts with thiols to form reactive sulfenylthiosulfate intermediates. Another sulfhydryl-containing molecule may couple to this active group to create a disulfide linkage.

Protocol

1. The macromolecule containing sulfhydryl residues to be blocked or protected is dissolved in a buffer suitable for its individual stability requirements. The blocking process may be done on a purified protein or during the early stages of a purification process to protect sulfhydryl-active centers from oxidation. PBS buffers containing 1 m*M* EDTA work well.
2. Add sodium tetrathionate to obtain a final concentration of 10 m*M*.
3. React for 1 h at room temperature.
4. Excess tetrathionate may be removed by dialysis or gel filtration.
5. To remove the sulfenylthiosulfate blocking group, add a 300-fold excess of DTT over the amount of blocked sulfhydryls present. Alternatively, add DTT to obtain a 0.01–0.1 *M* final concentration. Cysteine also may be utilized to regenerate some enzymes to full activity.
6. Incubate for 2 h at room temperature.
7. For removal of excess DTT, a protein of molecular weight greater than 5000 may be isolated by gel filtration using Sephadex G-25. To maintain the stability of the exposed sulfhydryl groups, include 10 m*M* EDTA in the chromatography buffer. The presence of oxidized DTT can be monitored during elution by measuring the absorbance at 280 nm. The protein should elute in the first peak and the DTT reaction products in the second peak.

Ellman's Reagent

Ellman's reagent, 5,5′-dithio-bis-(2-nitrobenzoic acid), or DTNB, is a compound useful for the quantitative determination of sulfhydryls in solution (Ellman, 1958, 1959). The disulfide of Ellman's reagent readily undergoes disulfide exchange with a free sulfhydryl to form a mixed disulfide and release of one molecule of the chromogenic substance 5-sulfido-2-nitrobenzoate, also called 5-thio-2-nitrobenzoic acid (TNB). The intense yellow color produced by the TNB anion can be measured by its absor-

bance at 412 nm ($\varepsilon = 1.36 \times 10^4\ M^{-1}cm^{-1}$ at pH 8). Since each sulfhydryl present generates one molecule of TNB per molecule of Ellman's reagent, direct quantitation is easily done. This reagent has been used to measure the sulfhydryl content in peptides, proteins, and tissue samples (Anderson and Wetlaufer. 1975; Riddles *et al.*, 1979). See section 1.1.4.1 for the use of Ellman's reagent in the determination of sulfhydryl groups.

The same reaction between Ellman's reagent and the sulfhydryls of macromolecules can be used to block available —SH groups temporarily by the formation of a mixed disulfide bond. Treatment of a sulfhydryl-containing protein with an excess of Ellman's reagent blocks the accessible sulfhydryls with the TNB group, allowing chemical reactions to be done on other functional groups. Studies have shown that the rate of Ellman's reaction with the sulfhydryl groups in proteins is dependent on their accessibility (Damjanovich and Kleppe, 1966; Colman, 1969). The addition of a disulfide reducing agent then cleaves the TNB group and regenerates the free sulfhydryl. Enzymes containing sulfhydryls in their active sites may be reversibly blocked using this technique to preserve activity after modification or conjugation. Deblocking then restores catalytic activity in most instances.

Protocol

1. Dissolve the protein to be blocked at a concentration of 1–10 mg/ml in 0.1 *M* sodium phosphate, pH 8.
2. Dissolve the Ellman's reagent at a concentration of 4 mg/ml in 0.1 *M* sodium phosphate, pH 8.
3. Mix the protein solution with an equal volume of the Ellman's reagent solution and react for 15 min at room temperature.
4. Purify the modified protein from excess Ellman's reagent and reaction by-products by dialysis or gel filtration. A measurement of sulfhydryl content may be done by reading the absorbance of the modification reaction at 412 nm ($\varepsilon = 1.36 \times 10^4\ M^{-1}cm^{-1}$) versus a series of sulfhydryl standards treated in the same manner (e.g., cysteine).

To deblock the TNB-modified sulfhydryl residues, treat the protein with an excess of DTT according to the protocol described in section 1.1.4.1, DTT.

Dipyridyl Disulfide Reagents

The similar reagents 4,4′-dipyridyl disulfide (Grassetti and Murray, 1967) and 2,2′-dipyridyl disulfide (Brocklehurst *et al.*, 1974) react in a manner analogous to that of Ellman's reagent, both forming pyridyl disulfide bonds with free sulfhydryls and releasing a molecule of either pyridine-4-thione or pyridine-2-thione, respectively (Fig. 103). Both leaving groups are measurable spectrophotometrically at 324 nm (pyridine-4-thione) or 343 nm (pyridine-2-thione) to quantify the amount of sulfhydryl modification. The reagent 2,2′-dipyridyl disulfide is useful for creating sulfhydryl-reactive cross-linking agents, such as SPDP (Chapter 5, Section 1.1). Both reagents may be used to block sulfhydryl groups temporarily in macromolecules or to activate —SH groups for coupling to another sulfhydryl-containing molecule. The pyridine disulfide-modifying group can react with a sulfhydryl to form a disulfide

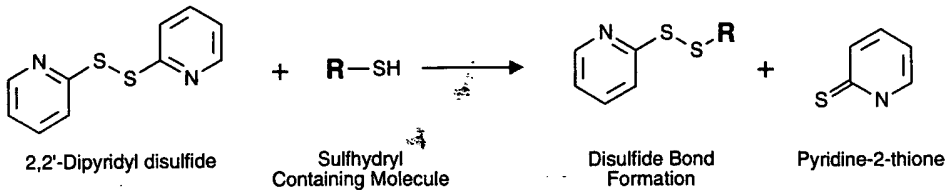
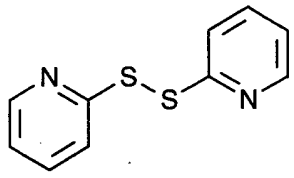
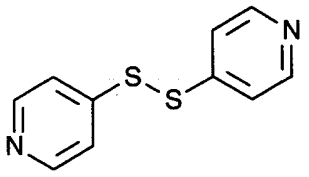


Figure 103 2,2′-Dipyridyl disulfide reacts with thiols to form an active pyridyl disulfide intermediate.

linkage. The pyridine disulfide also may be cleaved with an excess of disulfide reducing agents, such as DTT, making it a reversible blocking agent.



2,2'-Dipyridyl disulfide



4,4'-Dipyridyl disulfide

Unfortunately, 2,2′-dipyridyl disulfide is relatively insoluble in aqueous buffers. The use of this compound to modify molecules usually involves prior dissolution in an organic solvent such as acetone and then performing the blocking reaction in an aqueous/organic mixture. Many proteins will not tolerate high concentrations of organic solvents without precipitation.

The 4,4′-dipyridyl disulfide can be used in aqueous solutions, but it has been found that modification of proteins with this reagent yields rapid disulfide bond formation. Only when 2-iminothiolane is used in tandem with 4,4′-dipyridyl disulfide can 4-dithiopyridyl groups be introduced into proteins (King *et al.*, 1978) (see Section 4.1). This is due to disulfide interchange reactions predominating without the addition of 2-iminothiolane.

For one-step methods, the use of Ellman's reagent (previous section) to yield a similar reversible sulfhydryl blocking group is probably a better choice with protein molecules.

5.3. Blocking Aldehyde Groups

Aldehyde groups are useful in facilitating modification or conjugation reactions, easily forming secondary amine linkages with amine-containing molecules in reductive am-

ination procedures or hydrazone linkages with hydrazide-containing molecules. Macromolecules modified to contain aldehyde groups for use in these reactions (see Section 4.4) should be treated after conjugation to remove any excess formyl functionalities. The blocking step prevents subsequent nonspecific interactions when a conjugate is used in assay or targeting applications.

Reductive Amination with Tris or Ethanolamine

The simplest method for blocking aldehyde functionalities involves reductive amination with a small amine-containing molecule. The best such blockers do not have extra functional groups that may create additional sites of reactivity after blocking. Tris and ethanolamine are ideal in this regard. They both contain primary amines that readily react with aldehydes in the presence of a reductant, and they both possess relatively inert hydroxyl groups that maintain hydrophilicity after coupling. Reductive amination (Chapter 1, Section 5.3 and Chapter 3, Section 4) facilitated by the use of sodium cyanoborohydride can quickly block residual aldehyde groups and transform them into unreactive hydroxyls of low nonspecific binding potential (Fig. 104).

Protocol

1. Dissolve the macromolecuole containing aldehydes to be blocked (i.e., a glycoprotein that has been oxidized with sodium periodate to create formyl groups) at a concentration of 1–10 mg/ml in 0.1 *M* Tris buffer, pH 8. Alternatively, dissolve the macromolecule in 0.1 *M* sodium phosphate containing 0.1 *M* ethanolamine, pH 8. The use of other buffers having a pH between 7 and 10 will work as well, but the Tris or ethanolamine concentrations should be maintained in high excess to block efficiently all the aldehyde residues.
2. Add 10 µl of 5 *M* sodium cyanoborohydride in 1 *N* NaOH (Aldrich) per milliliter of the macromolecule solution volume prepared in (1). *Caution: Highly toxic compound. Use a fume hood and be careful to avoid skin contact with this reagent.*

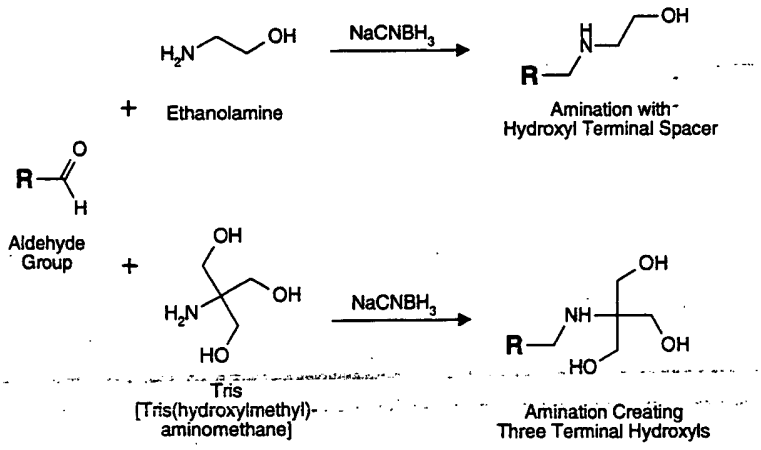


Figure 104 Aldehyde groups may be blocked with Tris or ethanolamine using a reductive-amination process.

3. React for 15 min at room temperature.
4. Purify the derivatized macromolecule by dialysis or gel filtration using a buffer suitable for the nature of the substance being modified.

5.4. Blocking Carboxylate Groups

The presence of unwanted carboxylate groups in macromolecules may be easily blocked by the use of a small amine-containing molecule coupled via the carbodiimide procedure (Chapter 3, Section 1).

Tris or Ethanolamine plus EDC

Tris or ethanolamine are excellent choices for blocking procedures involving carboxylic acid groups, since they contain hydrophilic hydroxyls that mask the carboxylate and create an inert modification with low nonspecific binding potential. Using the water-soluble carbodiimide EDC to facilitate this reaction, the carboxylate is activated by forming an intermediate *O*-acylisourea. The amine-containing compound then reacts with this active species to create a stable amide linkage (Fig. 105).

Protocol

1. Dissolve the macromolecule containing carboxylate groups to be blocked at a concentration of 1–10 mg/ml in 0.1 *M* MES, pH 4.7, containing 0.1 *M* Tris or ethanolamine. Other conditions may be used to perform this reaction. See Chapter 3, Section 1 for further details.
2. Add 10 mg of EDC per milliliter of the solution prepared in (1).
3. React for 2–4 h at room temperature.
4. Purify by gel filtration or dialysis.

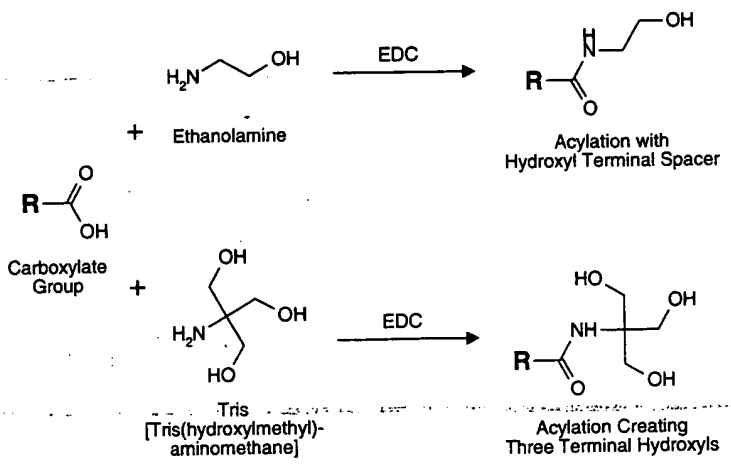


Figure 105 Carboxylate groups may be blocked with Tris or ethanolamine using a carbodiimide-mediated process.